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ANDREA FAEDO

IDENTIFICATION AND CHARACTERIZATION OF GENES PREFERENTIALLY EXPRESSED IN EMBRYONIC TELENCEPHALON AND CNS STEM CELLS

PhD Thesis submitted in partial fulfilment of the requirements of the Open University for the degree of Doctor of Philosophy in Molecular and Cellular Biology

March 2004

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Declaration

This thesis has been composed by myself and has not been used in any previous application for a degree. Results here presented were obtained by myself. Throughout the text I use both "I" and "We". Both forms are used interchangeable, and "We" does not mean other than "I", thus "We" does not necessarily mean that more people contributed to the result or discussion. All sources of information are acknowledged by means of reference.

Part of this work has been submitted for publication to the journal Mechanisms of Development.

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SUMMARY

One of the major goals in developmental neurobiology is to unravel the molecular programs controlling telencephalic development and neural differentiation. The complexity of brain cell types and circuits is reflected in the complexity of gene expression patterns in the brain. It is believed that perhaps a third to half of all genes are largely or exclusively dedicated to directing development, maintenance and functioning of the brain. In mammals, formation of the complex brain structure occurs over the long period of prenatal development. During this period neural progenitor cells must be instructed to undergo proper proliferation, migration, differentiation and connectivity.

The aim of my study was to identify genes, within a collection of novel genes preferentially expressed in the embryonic telencephalon, controlling such processes in the mammalian forebrain.

To this aim, as a preliminary step, an EST sequencing approach has been undertaken to catalogue and array the repertoire of genes represented in a subtractive library optimized to select rare or unique cDNAs preferentially expressed in the E14.5 mouse telencephalon (named "Telencephalic Embryonic Subtracted Sequences" (Porteus et al., 1992)). The hypothesis driving the production of such a library was that genes preferentially expressed during embryogenesis are likely to be specifically involved in the development of the telencephalon and in the biology of the neural progenitor cells. The selected transcriptome of 1026 unique cDNAs has been used to generate a unique microarray, and to perform gene expression profiling experiments on:

(i) mice mutant for transcription factors involved in forebrain development (Dlx1/2, Nkx2.1, Pax6, Ngn1/2),

(ii) in vitro cultured neural stem cells, committed neural progenitor cells (transient amplifying) and terminally differentiated neural cells.

The analysis of the resulting expression profiles has allowed the identification of novel genes that are candidates for playing a major role in neurogenesis and telencephalic development.

The differential expression identified with the *Tess* microarray has been validated using RNA *in situ* hybridization on embryonic tissue. Two novel genes (corresponding to *Tess* 28.8E and *Tess* 31.5E) have been found to be specifically down regulated in Dlx1/2 -/- subpallium (-46,51 fold for 28.8E; -6,44 fold for 31.5E), and up regulated in Pax6 -/- (4,34 for 28.8E; 3,15 for 31.5E) and Ngn1/2 -/- (9,02 for 28.8E; 5,04 for 31.5E) pallium.

The microarray experiments on neural stem cells allowed the identification of a selection of genes putatively involved in the process of self-renewal, lineage commitment and differentiation. Some of these genes have been analysed by RNA *in situ* hybridizations and demonstrated interesting restricted expression patterns in the developing telencephalon.

INTRODUCTION

Formation of the CNS

Gastrulation

Gastrulation is the process of highly coordinated cell and tissue movements whereby the position of the blastula cells, which were established during cleavage, are dramatically rearranged. During gastrulation, these cells are given new positions and new neighbours, and the multilayered body plan of the organism is established. The cells that will form the endodermal and mesodermal organs are brought inside the embryo, while the cells that will form the skin and nervous system are spread over its outside surface. Although the patterns of gastrulation vary enormously throughout the animal kingdom, there are only a few basic types of cells movements. Gastrulation usually involves some combination of the following types of movements:

- Invagination: the infolding of a region of cells
- Involution: the inturning of a region of cells from the surface layer to the interior of the embryo
- Delamination: the splitting of one cellular sheet into two more or less parallel sheets
- Epiboly: the movement of epithelial sheets that spreads to enclose the deeper layers of the embryo

At the beginning of Gastrulation, epiblast cells located at one region around the circumference of the egg cylinder adjacent to extraembryonic ectoderm undergo an epithelial-to-mesenchymal transition to form a transient embryonic structure called *the primitive streak*. At the anterior end of the primitive streak of the gastrulating mouse embryo lies the so-called *node*. The node has a bilaminar organization with a dorsal cell and a ventral cell layer. The mammalian embryo appears to have two signalling centers: the node ("the organizer") and the *anterior visceral endoderm* (AVE). While the node

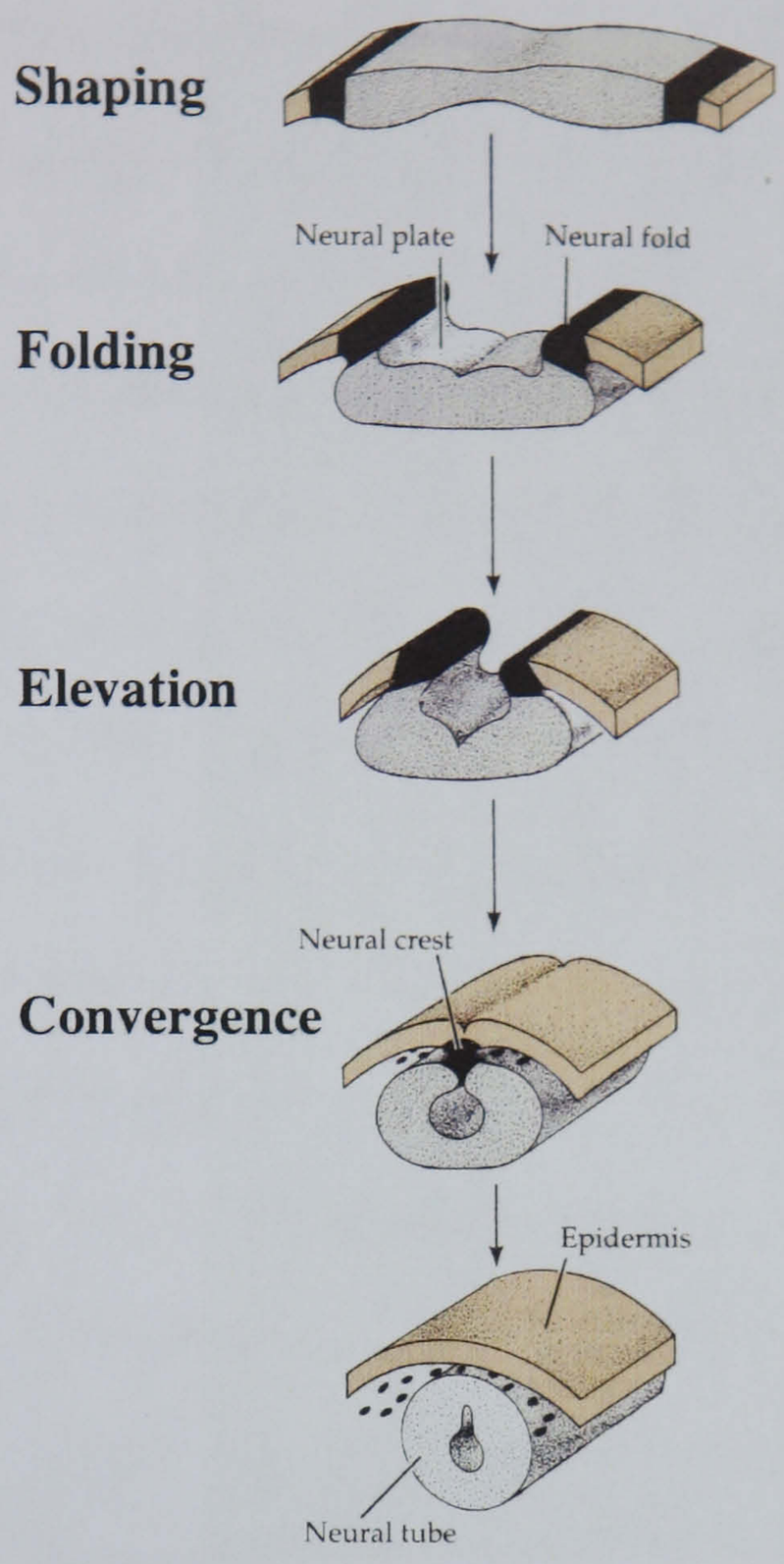
appears to be responsible for the organization of most of the body, these two signalling centers work together to form the forebrain (Bachiller et al., 2000). The node produces Chordin and Noggin, while the AVE expresses several genes that are necessary for head formation, including the transcription factors Hesx-1, Lim-1, and Otx-2, and paracrine factor Cerberus.

Gastrulation is the embryonic process leading to the generation of the three primary germ layers of the embryo, namely the endoderm, mesoderm and ectoderm. The endoderm forms the gut, associated organs, and the lungs. The mesoderm gives rise to two tissues in the embryo, the mesenchyme and the notochord. The latter plays a central role in the induction of the overlying neural tissue and is the precursor of the vertebrate column. The musculature, urogenital system and vascular system are also derived from mesoderm. The ectoderm forms the outer epithelium of the body and the neural plate, from which the nervous system develops.

Neurulation

The neural plate is committed to become neural tissue by a process named neural induction: this involves signals sent from a specialized mesodermal structure, the notochord. Soon after neural induction, the neural plate begins to fold to a neural groove, which fuses at its dorsal-most part to form the neural tube: this process, named neurulation, occurs in four stages (Fig 1).

Fig. 1



Schema illustrating the major events occurring in the neural tube formation.

(i) First, formation of the neural plate is initiated early in embryogenesis as ectoderm thickens, due to the induction by the chordamesoderm and rostral endoderm. During this stage, the ectodermal cells of the forming neural plate increase in height, undergo pseudo stratification and begin to express unique molecular markers. (ii) Second, shaping of the neural plate begins shortly after the neural plate forms. During this stage, the neural plate undergoes rostrocaudal lengthening, mediolateral narrowing and further apico-basal thickening, except in the midline where it becomes anchored to the underlying notochord and neuroepithelial cells shorten and become wedge-shaped. (iii) The third stage of neurulation is the bending of the neural plate, that involves the formation of hinge points, formation of the neural folds, and folding of the neural plate. The hinge points, one median and two dorsolateral, consist of areas of neural plate attached to adjacent tissues. The median hinge point (MHP) is attached to the prechordal plate, and the paired dorsolateral hinge points (DLHP) are attached to adjacent epidermal ectoderm. After the MHP forms, the neural plate on each side undergoes dorsal elevation around the longitudinal axis centered at the midline furrow. This results in the formation of the neural groove, an initially V-shaped space extending the length of the neural plate, flanked bilaterally by the incipient neural folds. (iv) The fourth stage is the fusion of the neural folds. The detached neuroepithelial layers from both sides fuse together deep to the epidermal ectoderm, establishing the roof plate of the neural tube.

The cavity of the neural tube gives rise to the ventricular system of the central nervous system, while the epithelial cells that line the walls of the neural tube (neuroepithelium) generate all the neurons and glial cells. While neurulation folds the neural plate into the neural tube, rapid cell divisions expand its surface area where individual regions expand differentially to give rise to the various specialized regions of the mature central nervous system. There are a group of specialized migratory cells that emerge from the dorsal region of the neural tube soon after its closure: the neural crest cells which migrate to generate a

wide variety of tissues, including sensory and autonomic neurons in peripheral ganglia, melanocytes in the skin, and connective tissues of the face.

Induction of the telencephalon

During gastrulation and early neurulation, the Organizer (Hensen's node) secretes molecules responsible for the neural induction of the ectoderm. Three main candidates have emerged as neural inducers: Noggin, Follistatin, and Chordin. The induction occurs by preventing the ectoderm becoming epidermis and by inducing it to become neural ectoderm instead.

The telencephalon derives from the rostral margins of the neural plate; telencephalic precursors are situated rostral and lateral to prospective eye tissue, located rostrally to the diencephalic territory. Thus the telencephalic anlage is under the influence of signalling pathways that regulate both the anterior-posterior (AP) and dorsal-ventral (DV) patterning of neural tissue. Mutations affecting the function of the embryonic organizer directly or indirectly alter AP patterning of the neural plate and lead to specification of forebrain fates. The gene *bozozok* (*boz*) encodes for a homeodomain protein required for a correct neural induction in zebrafish (Fekany et al., 1999; Koos and Ho, 1999). Embryos homozygous for mutations in the *boz* gene exhibit a phenotype in which anterior neural plate fates are variably reduced or lost. This is due to the impaired antagonism of Bmp and WNT signalling in the organizer. Forebrain fates can be rescued in *boz* null embryos by expression of Wnt antagonists, suggesting that *boz* normally negatively regulates the Wnt pathway and that Wnt signalling inhibits forebrain fates. Suppression of Wnt signalling is a prerequisite for telencephalic development. Moreover, experiments in frog embryos have suggested that blocking Nodal activity is also a requirement for induction of the head (Piccolo et al., 1999). The Nodal related protein Squint (Sqt) is required for development of organizer tissue, and embryos lacking both Boz and Sqt, have considerably less CNS

tissue and the telencephalon is absent. Analysis of zebrafish embryos lacking Nodal activity has confirmed that nodal signalling suppresses telencephalic development by blocking anterior neural plate fates (Fode et al., 2000; Gritsman et al., 1999). Studies in fish and frogs support a model of early neural patterning in which induced neural tissue will develop anterior character unless exposed to posteriorizing signals. Indeed, perhaps in all vertebrates, the primary role for signals promoting forebrain development, may be through the negatively regulation of factors that would posteriorize the anterior neural plate. In mammals, a possible source of antagonists of posteriorizing activity is the anterior visceral endoderm (AVE). This structure produces signals (i.e. Cerberus) that are antagonists of Wnt, Bmp, and Nodal proteins (Piccolo et al., 1999). The AVE is the extra-embryonic tissue that underlies the future neural plate, and several mutants that lack genes that are normally expressed in the AVE (like Lim1 and Otx2) fail to develop anterior structures, including the forebrain.

The anterior neural ridge

Ablation, transplantation, and *in vitro* explant studies in mice and fish have suggested that subsequent to neural induction, cells at the rostral margin of the neural plate (the anterior neural ridge) play an important role in the development of the telencephalon (Houart et al., 1998; Shimamura and Rubenstein, 1997). Telencephalic development is impaired when cells at the margin of the prospective neural plate are removed at gastrula stage in fish embryos, and these same cells express telencephalic genes when transplanted to more caudal regions of the neural plate. Furthermore, mouse neural plate explants in which the anterior neural ridge is removed fail to express the telencephalic marker Bf1, again supporting the notion that one or more signals from the margin of the neural plate promote telencephalic development (Shimamura and Rubenstein, 1997). It has been demonstrated that Fgf8 expression is necessary and sufficient to regulate Bf1 expression in vitro. It is in fact expressed in cells at the margin of the neural plate and later on at the midline of the

telencephalon (Shimamura and Rubenstein, 1997). It is, however, expressed too late to be the primary inducer of the telencephalon.

The hedgehog pathway and ventral telencephalic development

Dorsoventral patterning of spinal cord, hindbrain, and midbrain levels of the neural tube is regulated by the notochord, an axial mesendodermal structure that is located just ventral to the medial neural plate. Anteriorly to the notochord and ventrally to the prosencephalon lies the prechordal plate, which is likely to be involved in the patterning of the prosencephalic neural plate. Sonic hedgehog (Shh) is secreted by both the prechordal plate and notochord and is a potent patterning signal of the ventral neural tube.

The ventral telencephalon (subpallium) consists of the striatal (lateral ganglionic eminence), pallidal (medial ganglionic eminence), and anterior entopeduncular - preoptic area primordia, as well as parts of the septal and amygdaloid anlages. For each of these primordia the progenitor cells express a distinct combination of regulatory genes that defines the molecular identity of these structures. Cells contributing to the subpallium begin to express several specific genes at the neural plate stage (for example Otx2 and Vax1), while restricted expression of other subpallial genes is delayed and follows neurulation. Several lines of evidence suggest that SHH has an essential role in ventral telencephalic development.

Shh is a member of the Hedgehog family of secreted signalling protein implicated in a wide variety of developmental processes. Mice, fish and humans that have defects in Shh signalling lack ventral telencephalic structures (Chiang et al., 1996; Muenke and Beachy, 2000). Shh in fact promotes ventral telencephalic identity, and for instance, ectopic expression of Shh in mice and fish dorsal telencephalon induces ventral telencephalic markers such as Nkx2.1, Gsh2, and Dlx2 (Corbin et al., 2000; Gaiano et al., 1999). Patched is a component of the Shh receptor complex and functions as a negative regulator of Shh signalling (Kalderon, 2000). In Patched 1 mutant mice, expression of Nkx2.1 is expanded,

consistent with the notion that localized activity of Shh in the ventral telencephalon normally induces this gene (Goodrich et al., 1997). The Gli family of transcription regulators are downstream effectors of Shh signalling (Ruiz i Altaba, 1999), but there is no clear evidence for a requirement for Gli proteins in the establishment of ventral telencephalic territories, suggesting that Shh signalling may be mediated by others transcription factors. Several other factors downstream of Shh are key for the subdivision of ventral telencephalon including Nkx2.1 (Kimura et al., 1996), Gsh2 (Corbin et al., 2000), and Pax6 (Stoykova et al., 2000). Nkx2.1 mutants have a ventral-to-dorsal transformation of the ventral telencephalon, and cells fated to form MGE are transformed to an LGE identity. Once the telencephalic vesicles begin to evaginate, Nkx2.1 expression is induced in MGE, AEP/POA, and part of the septal primordia. Soon thereafter, the progenitor zones of the AEP/POA express Shh and the homeobox gene Isl1, suggesting that this gene contributes to the unique developmental features of this region.

A related phenotype is observed in mice carrying mutations in the Gsh2 homeobox gene, which is normally expressed in the VZ and SVZ of both the LGE and MGE. In these mutants, there is a down regulation of Dlx2 and an ectopic expression of the pallial markers in part of the LGE, indicating a pallial dorsalization of this region of the subpallium (Corbin et al., 2000; Yun et al., 2001). The resulting abnormal striatum is due to a misspecification of the LGE, and Gsh2 appears to be required for the repression of cortical specification genes in the subpallium.

Complementary to the Gsh2 and Nkx2.1 mutant phenotypes, Pax6 mutant mice express subpallial genes within the pallium indicating a ventralization of the dorsal telencephalon (Stoykova et al., 2000; Toresson et al., 2000; Yun et al., 2001). Mice lacking both Pax6 and Gsh2 show milder phenotypes than single mutants (Toresson et al., 2000), confirming that reciprocal regulatory interactions between these two genes mediate the DV patterning at the pallial/subpallial boundary. Expression of Pax6 is also restricted to the ventricular

zone of the LGE, and may contribute to the specification of the LGE in part through the repression of MGE specific genes. Accordingly, in Pax6 mutants the expression of MGE markers (e.g. Nkx2.1) expands dorsally into the LGE.

These data suggest that homeobox gene activity mediates the telencephalic DV patterning and establishes boundaries between regional subdivisions.

Fgf signalling also appears to regulate the patterning of ventral telencephalic structures. For instance, in fish, Nkx2.1 expression is reduced and ventral telencephalic midline tissue is disrupted in mutant embryos lacking Fgf8 function (Shanmugalingam et al., 2000).

Dorsal telencephalic development

Mutations in several transcription factors result in dorsal-to-ventral transformations within the telencephalon. Mouse Gli3 mutants lose expression of many dorsal markers, and structures such as the hippocampus, and LGE markers spread into the cerebral cortex (Theil et al., 1999). The expression of the homeobox Emx2 gene is also reduced in this mutant. Emx2 is normally expressed in the caudal/dorsal cortical areas, and it has been proposed that graded Emx2 activity may confer the regional identity within the cortex (Bishop et al., 2000; Mallamaci et al., 2000). Complementarily, cortical Pax6 expression is higher rostrally and ventrally, and its loss leads to expansion of the caudal cortical domains at the expense of rostral ones. Graded opposing activities of the homeodomain proteins PAX6 and EMX2 may therefore generate positional identities within the dorsal telencephalon and contribute more generally to the overall regional subdivision of the telencephalon.

Other families of transcription factors also have a profound influence in the development of this region of the brain. For instance, a striking dorsal-to-ventral fate transformation occurs in mice with altered expression of members of the bHLH family of transcription factors (Fode et al., 2000). In both vertebrates and invertebrates, bHLH proteins regulate neurogenesis and influence neuronal identity (Ross et al., 2003). In the telencephalon

Mash1 is expressed ventrally, while two neurogenin (Ngn1 and Ngn2) genes are expressed dorsally. In the Ngn2 or Ngn1 - Ngn2 double mutants there is an ectopic induction of ventral molecular markers such as Mash1 in the cerebral cortex, indicating that Ngn activity promotes dorsal telencephalic development by suppressing ventrally expressed genes (Fode et al., 2000). Other experiments demonstrate that Mash1 when ectopically expressed in the pallium is both required and sufficient to confer ventral telencephalic identity to the neurons (Fode et al., 2000).

There is evidence that the same patterning mechanisms that regulate lateral patterning in the neural plate also participate in the regional specification of the cortex. Secreted proteins of the TGF-β (BMP, GDF), WNT, and FGF families have been in fact implicated in the dorsal patterning of the telencephalon and its subsequent regionalization. TGF-β and WNT genes are predominantly expressed in the dorsalmedial region of the telencephalon, while Fgf8 expression is highest in the rostral midline. After neural tube closure, a domain of Bmp2, 4, 6, and 7 coexpression defines the dorsomedial region of the telencephalon. As development proceeds, expression of Bmp4, 5, 6, and 7 identifies the prospective hippocampus, fimbria, and choroid plexus, whereas Bmp2 expression become restricted to the developing dentate gyrus of the hippocampus (Furuta et al., 1997).

Subpallial ectopic Bmp activity represses ventral telencephalic markers (Nkx2.1 and Dlx2), and leads to decreased proliferation, increased apoptosis, and holoprosencephaly (Furuta et al., 1997; Golden et al., 1999). Implantation of Bmp4 or Bmp5 soaked beads into the chicken forebrain induces the expression of dorsal markers and repression of ventral markers (Golden et al., 1999). The generation of mice bearing mutations in either Bmp2, 4, 5, 6 and 7 has proven to be insufficient to understanding their role in forebrain patterning, probably due to genetic redundancy as suggested by their overlapping expression patterns. In agreement with this hypothesis, Bmp5/Bmp7 double mutants exhibit delayed closure of the rostral neural tube and hypoplasia of the telencephalic

vesicles (Solloway and Robertson, 1999). Several transcription factors have been implicated in the transduction of BMP signals. The expression of BMPs in the dorsomedial telencephalon correlates with high levels of Msx1 expression, and in vitro experiments have shown that BMPs can induce Msx1 expression (Furuta et al., 1997).

The WNT family of secreted proteins appear to cooperate with BMPs in the patterning of the pallial telencephalon. There is growing evidence that WNT proteins specify dorsal telencephalic fates and expand dorsal progenitor cell populations. Like Bmp genes, Wnt family members (e.g., Wnt2a, 3a, 5a, 7a, 7b, and 8b) are expressed in domains of the dorsal telencephalon. While some Wnt genes are broadly expressed in the telencephalon (Wnt7b), others are restricted to specific dorsalmedial domains (e.g., Wnt3a in fimbria). Loss of function mutations of the gene Wnt3A and in the downstream effector Lef1 lead to a loss/reduction of the hippocampus (Galceran et al., 2000). WNT signals are mediated by the FRIZZLED family of receptors, which act to stabilize β-catenin and propagate the signal intracellularly (Wodarz and Nusse, 1998). Increased β-catenin concentration leads to the activation of Lef/Tcf transcription factors, which mediate many of the final responses of WNT signaling. Inhibition of WNT signalling is mediated by secreted factors of the Frizzled-related proteins (SFRP) family, which have an amino-terminal domain highly homologous to the ligand binding domain of Frizzled proteins. Expression of SFRPs is induced by Wnt genes, perhaps to fine-tune the spatial and temporal patterns of Wnt activity. For example high levels of SFRP2 expression are found at the pallial-subpallial boundary, suggesting that this protein may play a prominent role in the patterning of this region through the modulation of WNT signals from the surrounding structures (Kim et al., 2001).

DV regionalization and migration of neurons

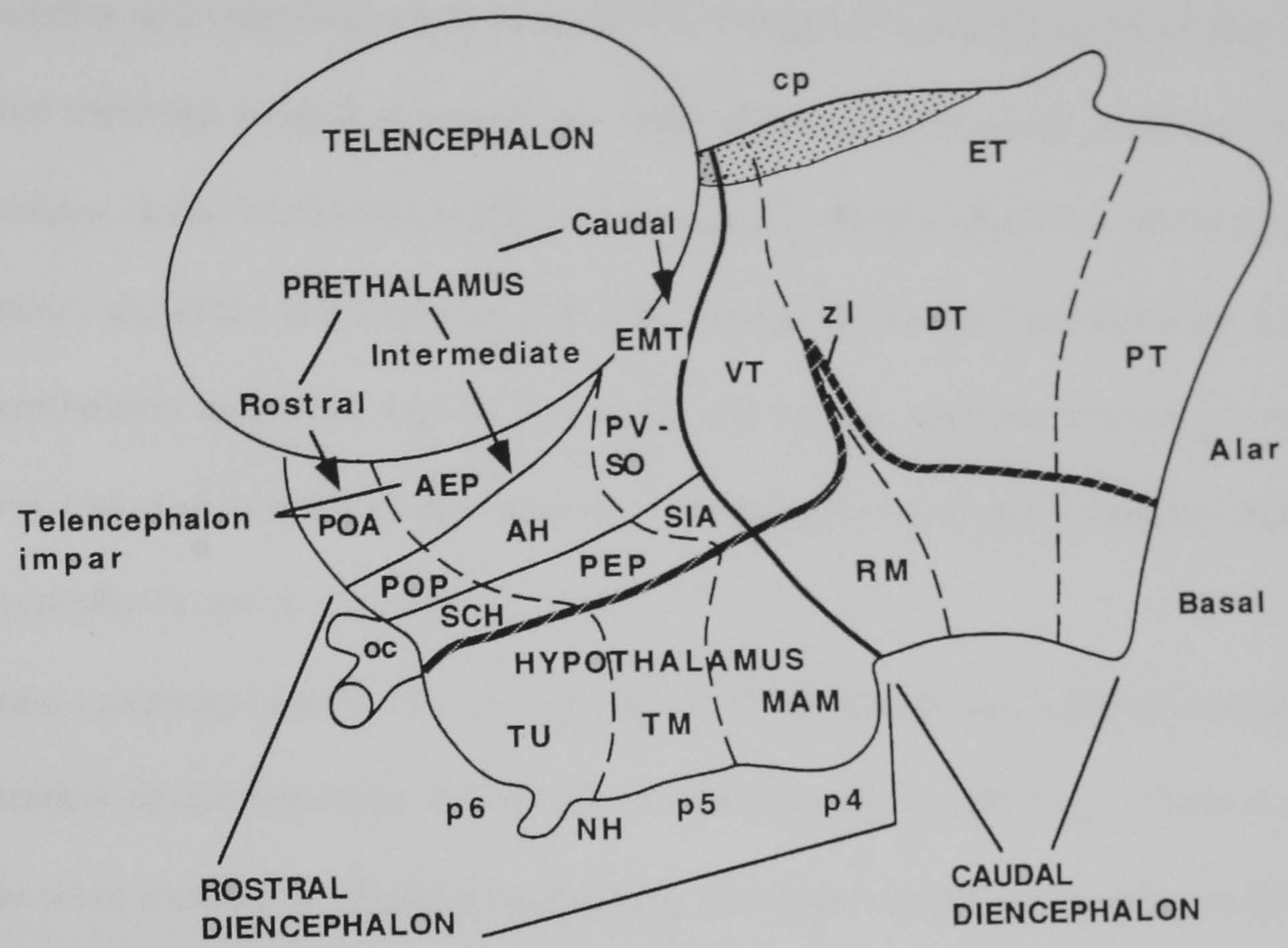
While distinct patterning mechanisms regulate the specification of cerebral cortex and basal ganglia primordia, recent studies have revealed unexpected interdependence between

these structures. In fact, in the basal ganglia anlage are produced both projection neurons, that migrate radially to their final locations (within the basal ganglia), and interneurons, which mainly migrate tangentially to the cortex. An important aspect of early DV patterning is the specification of progenitor cells fated to give rise to neurons synthesizing specific neurotransmitters: glutamatergic neurons are generated in the cortex, GABAergic neurons in the MGE and LGE, cholinergic neurons only in the MGE. The projection neurons generated in the different structures follow radial migration and maintain the positional information essential for the generation of topographic connectivity maps.

Anatomy of the forebrain

The early regional specialization of the central nervous system is imposed by the inductive activity of the underlying mesoderm. The caudal part of the neural tube gives rise to the spinal cord. The rostral neural tube gives rise to the three vesicles: *forebrain*, *midbrain* and *hindbrain*. The primitive forebrain, or primary prosencephalon, soon divides into the diencephalon (caudally), and secondary prosencephalon (rostrally) (Fig 2).

Fig. 2



This schema highlights subdivisions in the secondary prosencephalon, sum of rostral diencephalon and telencephalon, and caudal diencephalon. Abbreviations: AEP, anterior entopeducular area; AH, anterior hypothalamus; DT, dorsal thalamus; ET, epithalamus; EMT, eminentia thalami; MAM, mammillary region; NH, neurohypophysis; OC, optic chiasm; POA, anterior preoptic area; POP, posterior preoptic area; PEP, posterior entopeduncular nucleus; PV-SO, paraventricular-supraoptic nucleus, PT, pretectum; SCH, suprachiasmatic nucleus; SIA, subincertal area; TU, tuberal region; TM, tuberomammillary region; VT, ventral thalamus (pre-thalamus); ZL, zona limitans.

The forebrain is derived from the anteriormost transverse domain of the neural plate. Tissues adjacent to and within the neural plate produce molecules that regulate regional specification and morphogenesis of the CNS. Prospective subdivisions of the brain are specified thorough several mechanisms. Anteroposterior patterning generates transverse subdivisions, dorsoventral patterning generates longitudinally aligned domains (floor plate, basal plate, alar plate, and roof plate). Finally, regionally distinct molecular profiles of the neuroepithelium and local signals from adjacent tissues regulate the outgrowth of the bilateral eye and telencephalic vesicles (secondarily also the olfactory bulbs), the neurohypophysis, and the epiphysis.

Forebrain morphogenesis is the complex result of the spatial and temporal control of cell proliferation, death, migration, axonal growth, and changes in cell shape. There is evidence that the same molecules regulating patterning also regulate precursor cell proliferation in the forebrain. For example, WNT signalling is implicated in positively regulating the proliferation through c-myc and cyclin D1 (Shtutman et al., 1999; Tetsu and McCormick, 1999). Members of the TGF-β superfamily have instead the opposite effect: for example, local application of beads containing BMPs inhibits proliferation and induces local apoptosis both in telencephalic explants (Furuta et al., 1997) and within the telencephalon in vivo (Golden et al., 1999). FGF8 signaling is also implicated in the control of telencephalic growth: reduction of Fgf8 expression at the rostro-dorsal midline of the telencephalon results in severe hypoplasia of the rostral telencephalon (Storm et al., 2003). Recent studies suggest that these molecules function together and that the complex interplay between them controls proliferation and apoptosis in the forebrain.

In order to organize the gene expression patterns in relation to the developmental morphological changes during prosencephalic development a model has been recently developed. The prosomeric model of forebrain organization is, in fact, instrumental for

addressing the putative role of novel gene in forebrain development on the basis of their expression patterns (Puelles and Rubenstein, 1993; Puelles and Rubenstein, 2003).

The concepts at the basis of the model are that the caudal diencephalon has three transverse subdivisions: the prosomeres p1-p3 (Puelles and Rubenstein, 1993; Puelles and Rubenstein, 2003). These contain the pretectal (p1), thalamic/epithalamic (p2), and prethalamic regions (p3), and each prosomeres has alar (dorsal) and basal (ventral) components. The transverse organization of the secondary prosencephalon is not yet defined in separate neuromeres but subdivided into different domains, which exhibit patterning singularities (Puelles and Rubenstein, 2003).

In the model the telencephalon is represented as a lateral and dorsal extension of the diencephalon from which the optic and telencephalic vesicles evaginate.

At late neural plate stage, most of the diencephalon is distinguished from the telencephalon by the lack of Foxg1 expression. The diencephalon consists of both basal and alar plate domains, where the basal plate is induced and patterned by SHH mediated signals and Gli zinc finger transcription factors. Dorsal patterning and regionalization is instead mediated by members of the TGF-β, WNT, and FGF families.

The caudal diencephalon

The pretectum (alar p1) is the most caudal forebrain region. The major structures of p1 are: the posterior commissure (it contains various pretectal nuclei involved in visual processing) and the dopamine-containing neurons that form part of the substantia nigra in basal p1. Rostrally to the pretectum lies the epithalamus and thalamus (alar p2). Its dorsal midline includes the epiphysis, the habenular commissure, and choroid plexus. The thalamus (or dorsal thalamus) lies rostral to the epithalamus and consists of an elaborate complex of nuclei that mainly project to the telencephalon (targeting both subcortical and cortical structures). These nuclei serve as relay centers for numerous pathways in various functional circuits. The sensory nuclei process somato-sensory and viscero-sensory

information (ventrobasal complex), visual input (dorso lateral geniculate nucleus), and auditory input (medial geniculate nucleus), which are relayed by these nuclei to the primary sensory areas of the isocortex. Other thalamic nuclei are part of the motor control system (ventral anterior and ventrolateral nuclei). The anterior and periventricular thalamic nuclei are part of the limbic system. The dorsal thalamic nuclei are instead part of the associative system that in humans is particularly developed and complex. The basal p2 is poorly understood, it contains a part of the dopaminergic neurons of the substantia nigra.

The p2/p3 limit appears as a fiber-rich gap between the dorsal and ventral-thalamus, and it is known as the *zona limitans intrathalamica*. The choroid plexus, a neuroepithelial specialization where the cerebrospinal fluid is produced, extends from p2 thorough p3 into the telencephalon.

Alar p3 consists of the ventral lateral geniculate nucleus, the reticulate thalamic nucleus and a periventricular *zona incerta* (the latter two nuclei contain many inhibitory neurons).

The rostral diencephalon

This term is used to refer the extratelencephalic part of the secondary prosencephalon (Puelles and Medina, 2002): its alar plate forms the prethalamus while the basal/floor plate contains the hypothalamic structures.

The rostral diencephalon mainly controls the inner viscera, neurohumoral functions, and internal homeostasis.

The caudal part of the hypothalamus includes the mammillary region, the subthalamic nucleus, the lateral hypothalamic area, and part of the posterior hypothalamus. The intermediate hypothalamus is represented by the tuberomammillary region. The corresponding alar or prethalamic area extends through the posterior and anterior entopeduncular area into the telencephalic stalk. The rostral hypothalamus corresponds to

the tuberal region and neurohypophysis. The rostral prethalamic alar plate continues through the posterior and anterior preoptic region into the telencephalic stalk.

The telencephalon: basic parts

The telencephalic hemispheres evaginate bilaterally from the dorsal most alar plate of the secondary prosencephalon. Rostrally, a thick median wall portion is the site where the telencephalic commissure develops. These are fibers interconnecting both vesicles: in mammals they correspond to the anterior commissure, hippocampal commissure, and corpus callosum.

There are two principal subdivisions of the telencephalic vesicles: the roof, or pallium, and the base, or subpallium.

The subpallium

The subpallium consists primarily of nuclei, the so-called basal ganglia (BG). In the pallium there are primarily cortical structures but also some pallial nuclei. The area occupied by the basal ganglia in the hemisphere differentiates and stops growing early, whereas the overlaying pallium is capable of prolonged surface growth. The increasing disproportion between these two parts leads to a characteristic morphogenetic deformation consisting of a progressive bulging and relative diminution in size of the sub-pallium, forced by anteroposterior and mediolateral expansion of the pallium. New anterior and posterior poles appear, forming the definitive frontal and occipital poles of the hemispheres. An outgrowth at the early forming anterior pole forms the olfactory bulb, which gradually becomes displaced. The basal ganglia are crossed by the radiating fibers of the internal capsule (bidirectional thalamocortical axons) and become secondarily subdivided into several portions.

The subpallium may be divided into a middle sector and two transitional domains at its rostral and caudal ends. The wall of the middle sector bulges into the ventricular cavity

early in development, forming two ridges called the medial and lateral ganglionic eminences (MGE and LGE); these transient structures are perforated by the major tract interconnecting the diencephalon with the telencephalon, the internal capsule. The mantle layer under the MGE matures earliest and gives rise to the globus pallidus (GP) or pallidum. The internal capsule fibers separate the GP from the periventricular pallidal derivates, which forms a curved complex called the bed nucleus of the stria terminalis. The stria terminalis, LGE, and MGE produce waves of neurons that migrate tangentially to invade the striatum and the whole cortical pallium.

The mantle layer of the LGE will become the striatum. In humans, the internal capsule partially divides the dorsal striatum into a portion called putamen and a periventricular portion called caudate nucleus.

The striatopallidal complex is integrated into a circuit that modulates the motor output of the motor and premotor cortex. The cortex projects excitatory axons to specific areas of the striatum complex. The striatum projects separately to the external and internal parts of the pallidum, where its effect is inhibitory.

The amygdala and the septum are located rostral and caudal to the striato/pallidal complex respectively and can be divided into pallial and subpallial parts according to the expression of genetic markers.

The basal ganglia (BG) constitute key brain structures for the motor functions, in particular in the planning, initiation and execution of movement, and are involved in a variety of non-motor functions, including those related to incentive and motivated behaviours. In a restrictive sense, the term basal ganglia refers to the striatal and pallidal components of the basal telencephalon that develop from the lateral and medial ganglionic eminences, respectively. The term, however, frequently includes the substantia nigra (SN), the ventral tegmental area (VTA) and the subthalamic nucleus (STN).

The subpallium generates projection neurons and local circuit neurons, which can be both GABAergic and cholinergic. Available evidence suggests that most, if not all, differentiating GABAergic neurons express the Dlx and Mash1 genes. Projection neurons largely follow radial migratory routes from the progenitor zone into the overlying mantle. The striatal projection neurons are thought to derive from the LGE (Anderson et al., 1997b), whereas most pallidal neurons appear to derive from the MGE (Sussel et al., 1999). In addition, the MGE and the adjacent AEO/POA probably give rise to the projection neurons of the cholinergic magnocellular basal forebrain complex. Cortical interneurons originate in the subpallium and follow two main tangential migrations, a superficial route from the MGE (Anderson et al., 2001; Lavdas et al., 1999; Sussel et al., 1999; Wichterle et al., 1999), and a deep pathway originating in the LGE (de Carlos et al., 1996; Pleasure et al., 2000).

The pallium

The pallium is a primary embryonic telencephalic structure, encompassing most of the telencephalic vesicle, and distinguished early on from the underlying subpallium by specific gene expression code. For instance, the prospective subpallium is marked by the expression of Dlx genes (Eisenstat et al., 1999; Liu et al., 1997), whereas, among other markers, Pax6 and Tbr1 mark the pallium. Both pallium and subpallium soon regionalize into sub-regions, which nevertheless keep their primary molecular hallmarks. In all vertebrates, the pallium seems to regionalize, on the basis of histochemical, gene expression and hodological data, into four territories: medial, dorsal, lateral and ventral pallial domains (Puelles, 2001). The medial pallium develops into the hippocampal allocortex (three layered), while the dorsal pallium forms the isocortex (six-layered), which is the part that expands more during mammalian evolution and is variably subdivided in mammalian species. The lateral and ventral pallial domains both give rise to portions of the olfactory cortex, and to nuclear pallial derivatives, placed near the subpallium, such as the

claustrum, the endopiriform nuclei, the lateral and basal part of the amygdala, a small portion of the septal nuclei, dorsally to the major subpallial part of the septum. The olfactory bulb, anterior olfactory areas, and the olfactory allocortex cover most of the nuclear parts of the pallium.

These pallial fields are though to be homologous across the taxa, and are relevant for understanding cortical development and evolution. The term "pallium" is more appropriate than the term "cortex", since it applies to homologous embryonic and adult pallial origin, independently of whether a cortical structure is already fully differentiated. The reptilian ancestors of mammals had already some sort of primitive cortical structure in each of the pallial sub-regions (Puelles, 2001). These structures, modified by increased cellularity, stratification and surface growth, later evolved into the avian and mammalian pallial cortex. The mammalian dorsal pallium evolved into the more complex and extensive, six-layered isocortex. The mammalian isocortex therefore is topologically an isolated "dorsal pallium" island, which lacks olfactory projections and is surrounded by lateral and medial allocortex and some pallial nuclei. The isocortex surface is proportionally smaller in anamniotes and in non-mammalian amniotes, and it acquires maximal extension in the gyrencephalic mammalian brains (as opposed to mammals having lissencephalic cortex, like the mouse). The other pallial regions show the same topological relationships, but lack the histogenetic differentiation characteristic of mammals.

The developing isocortex

Anatomy

As described above the cerebral neocortex is the most prominent region to emerge from the telencephalon. In the adult, the neocortex has six principal layers, numbered 1 (most superficial) to 6. The cortical layers have different neuronal subtypes, different patterns of afferent and efferent axonal connections, and express different sets of genes. At the stage

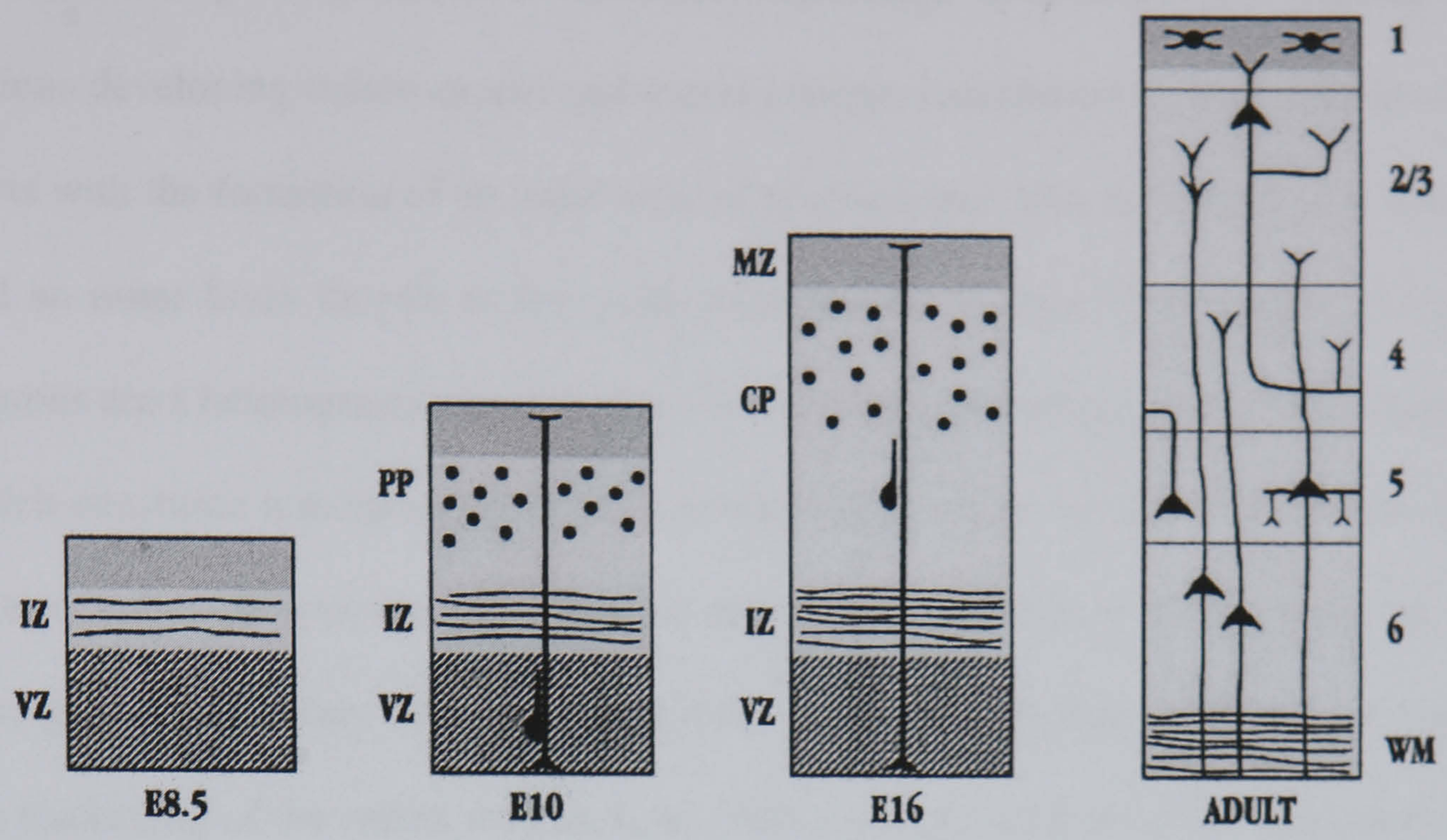
when the telencephalic vesicles bulge the cortical primordia consists of a thin germinal neuroepithelium, with mitotic spindles of dividing cells close to the ventricular surface. Subsequently three broad classes of cells are found in the neuroepithelium:

- postmitotic neurons,
- glial cells,
- progenitor cells.

From the dividing progenitor cells originate postmitotic neuronal precursors, which undergo migration and differentiation before becoming mature neurons. Gliogenesis occurs mostly after neurogenesis is complete and continues postnatally. The large pool of progenitor cells of the developing neocortex disappears in early postnatal life, although some progenitors remain in specific regions of the adult brain (see Neural stem cells-introduction).

Corticogenesis is the process leading to the formation of the six-layered cerebral cortex from the neuroepithelium of the dorsal telencephalon (Fig. 4).

Fig. 3



Corticogenesis. In early phases of development, neurogenesis is ongoing in the compact germinal zones lining the ventricles (left). Axons grow over this zone to form the intermediate zone (IZ). The next wave of postmitotic neurons migrates through the IZ to form the preplate (PP). Continued migration splits the PP into the marginal zone (MZ) and cortical plate (CP). Thereafter, successive waves of migration position cells within six layers.

There are temporal gradients of neuronal maturation with rostral and ventral cortical regions developing before caudal and dorsal regions. Lamination of the telencephalic wall starts with the formation of an inner layer of proliferating cells, the *ventricular zone* (VZ), and an outer layer known as the primordial plexiform layer or *preplate*. The preplate neurons are a heterogeneous population of cells that form a primitive cortical organization, which constitute a morphogenetic framework within which the main layers develop. This layer contains prospective Cajal-Retzius cells, which are born in the VZ early in cortical neurogenesis, and they begin to differentiate and migrate before other cortical layers. As the thickening of the cortex increases, an additional layer of low-density post-mitotic cells appears above the VZ, the *intermediate zone* (IZ) that eventually will contain the major afferent and efferent axonal tracts of the cerebral cortex (future white matter). Superficially to the IZ the preplate splits into a superficial and a deep layer, named *marginal zone* (MZ) and *subplate* respectively, which together form the so-called cortical plate (CP). The marginal zone will eventually form layer 1 of the adult cortex, and its principal neuronal cell type is the large horizontal Cajal-Retzius neuron, which are simple horizontal bipolar neurons in small rodents and lower mammals, but show more intricate morphological features in higher mammals with different cell shapes. Axons of neocortical Cajal-Retzius cells radiate within the lower part of the marginal zone and may extend for long distances. Their function is to regulate the formation of cortical layers, via a constitutive expression of reelin (Marin-Padilla, 1998). These cells, besides expressing reelin, express specific transcription factors such as Tbr1 (Hevner et al., 2001).

The subplate is a transient structure containing highly differentiated neurons fated to die postnatally, which has an essential role in the initial formation of connections. In the cortex, subplate cells attract cortical afferents, and may guide and pioneer some corticofugal pathways (Ghosh et al., 1990).

Together with the formation of the cortical plate, another layer becomes visible between the ventricular and intermediate zones, the *subventricular zone* (SVZ). This dense zone of dividing cells represents a secondary germinal layer constituted by cells produced in the ventricular zone. During embryogenesis, its thickness gradually becomes larger than the VZ, and the distribution of the cells is not radially ordered.

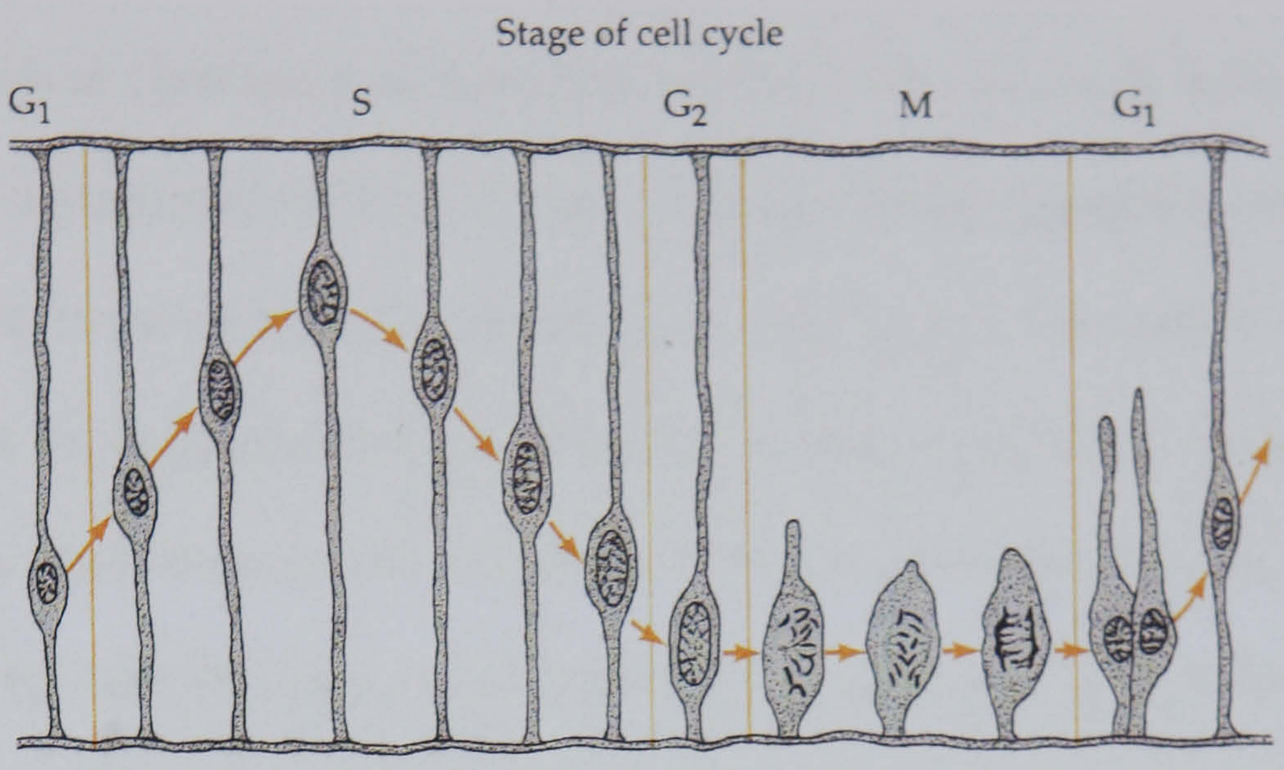
The cellular processes at the basis of the morphological changes of the telencephalic vesicles are: the generation of new neurons in the proliferative zones of the cortical wall, and their migration towards the pial surface, where they terminally differentiate.

Cell proliferation

The cell cycle of eukariotic cells can be divided into four successive phases: (i) M phase, during which the nucleus and cytoplasm divide, (ii) G1 phase, between the end of M phase and the onset of DNA synthesis, (iii) S phase, during which the DNA is replicated, and (iv) G2 phase, between the S phase and the next M phase. The length of the cell cycle varies enormously, depending on cell types and stage of development. Most of the variations are in the length of G1 phase. Some cells, such as neurons, arrest in G1 phase in a quiescent state known as G0. The lengths of the different phases of cell cycle can be experimentally measured by administering nucleotides analogs such as tritiated thymidine or bromodeoxyuridine (BrdU) that are both incorporated into newly synthesized DNA.

In cortical development the overall length of the cell cycle increases progressively, becoming nearly twice as long at the end of neurogenesis. During the cell cycle, the cell nuclei along the ventricle move intracellularly (Fig 3), such as in S phase the nuclei are away from the ventricular lumen, while in G2 they move back to the ventricular surface to undergo mitosis.

Fig. 4



Nuclear movements during cell cycle in VZ cell proliferation

It has been assumed, and recently shown (McConnell, 1995), that there are two types of cell division in the ventricular zone: symmetric and asymmetric. The symmetric cell division by vertical cleavage generates two daughter cells that both maintain stem cell properties. The asymmetric cell division by horizontal cleavage generates one daughter cell that migrates out to the cortical plate and one that remains as a stem cell in the ventricular zone. Thus, it is suggested that symmetric divisions are more common early in corticogenesis, when neurogenesis is greater. These observations suggest a relationship between the rates and the types of division in the ventricular zone and they raise two intriguing possibilities: (i) whatever regulates the proliferation rate may also regulate cleavage type, ensuring it is appropriate for the required rate; (ii) that the factors regulating proliferation act primarily on the types of cleavage.

At each mitotic division, the fate of each daughter cell, either re-entering or exiting the cell cycle, dramatically influences neocortical growth. Symmetrical founder cell divisions, which predominate early, yield more progenitors and lead to an exponential expansion of the VZ population. In contrast, asymmetrical divisions, which prevail during later stages of neurogenesis, lead to cell commitment and diversity of the cortical architecture. Critical parameters of cortical growth are, therefore, the duration of the early symmetrical division phase and the time and extent of the transition to the asymmetrical mode of division. It has been shown that the conversion between asymmetric and symmetric cell division is related to the rearrangement of the mitotic plate before division and that spindle movement during metaphase is a prerequisite for modulating the mode of division (Haydar et al., 2003).

A major question that remains open is the way region specific numbers of neurons, glial and progenitors cells are regulated during development

The switch to gliogenesis: glial cells.

Glial cells constitute the large majority of cells in the nervous system, and their main role is to support the development and function of neurons. In the adult CNS, the term glial

cells include oligodendrocytes, astrocytes, ependymal cells and microglia. The oligodendrocytes wrap themselves around the axons to provide the insulating myelin sheath, essential for neurons to transmit action potential at an appropriate speed. An oligodendrocyte extends many processes, each of which contacts, and repeatedly envelopes a stretch of axon, forming myelin.

Astrocytes also have many processes radiating from the cell body. These may end on the surface of neurons, on the external surface of the CNS, or on the endothelial cells of blood vessels. Ependymal cells line the central cavities of the CNS (the central canal of the spinal cord and the ventricles of the forebrain) formed after the neural tube closure, and contribute to the regulation of the chemical composition of both the CNS parenchyma and the cerebrospinal fluid. Microglia cells are the equivalent of the macrophages found in other organs. With the exception of microglia, glial cells and neurons have a common embryonic neuroectodermal origin. During development common progenitor cells must at some point follow a specific developmental pathway (gliogenic or neurogenic) leading to the differentiation into one of the various glial or neuronal cell types. It is well accepted that, although some glial cells appear early in cortical development, the vast majority are generated after neurogenesis is complete. In general, therefore, there is a switch during late gestation from the production of neurons to the production of glia cells. At this stage, once neuronal migration is complete there are two cell populations in the cortex that contribute to the glial lineage (i) the early generated radial glia cells which transform into astrocytes, and (ii) the cells of the secondary proliferative population in the subventricular zone, which are the major source of cortical glial cells, contributing to both astrocytes and oligodendrocytes.

Cell migration in the forebrain

The forebrain is one of the most evolved regions of the mammalian brain, and its extraordinary degree of organization reflects the complexity of the migratory movements taking place during embryogenesis. Moreover, any defects in neuronal migration during development lead to cortical dysplasias, epilepsy, and severe learning disabilities. Understanding how neural cells migrate is therefore essential to unravel the mechanisms underlying normal and pathological brain development.

Patterning and specification of the forebrain regions precedes cell migration. In particular the regional specification is obtained via a combinatorial expression of different transcription factors that, through mutually repressive interaction, establish boundaries between different progenitors zones; and only once the cells are specified, they set out and migrate to their final position in the forebrain mantle layers.

As in other CNS regions, two general types of migration have been identified in the forebrain: (i) radial migration, in which cells migrate from the progenitor zone towards the surface of the brain following the radial alignment of glial fibers; (ii) tangential migration, in which cells migrate orthogonally to the direction of radial migration between the ependymal layer and the pial surface.

Radial migration

The concept of radial organization of the neural tube originates from the principle that there is a point-to point relationship between the VZ and the pial surface due to the radial glia scaffold (Rakic, 1972). The radial glia cells arise throughout the neural tube during early development of the VZ, where these cells have their soma, while their processes span the wall of the neural tube and reach the pial surface anchoring to the basal membrane. During development of the cerebral cortex, radial glia processes serve as a scaffold to support and direct neurons during their migration, and once neurogenesis is complete,

radial glia cells retract their ventricular and pial attachments and differentiate into astrocytes. This narrow view of the role of these cells is now challenged by the evidence that they undergo mitosis and generate new neurons. Recent studies have in fact provided strong support for an active role as neuronal precursors cells in the ventricular zone, in addition to providing migration guidance (Gaiano and Fishell, 2002; Hartfuss et al., 2001; Malatesta et al., 2000; Noctor et al., 2002; Weissman et al., 2003). The most recent view suggests that newly generated cells in the VZ assume a bipolar shape and migrate along the radial fiber of the mother cell, which remains attached to the ventricular surface.

Radial glia cells have been observed in most regions of the mammalian brain during specific developmental periods, suggesting a pivotal role in constructing the nervous system. Radial glia cells have also a particular species-specific adaptation. For example, they have straight and short migratory pathway in rodents but in the larger primate cortex the glial fibers are longer and increasingly curved (Rakic, 1978). During evolution, the cerebral cortex expanded mostly in surface area rather than in thickness, and as a result cerebral hemispheres become increasingly more convoluted. Thus migratory neurons have to follow curvilinear pathways along the glial fibers rather than moving in a straight fashion to the closest site in the cortex. Therefore, radial glia cells are truly the cellular building blocks for the laminar and radial architecture of the primate cerebral cortex. Clones of newly generated neurons in the VZ use a common migratory pathway along the radial glia fascicle, through the intermediate and subplate zones, to settle within and along the same column in the cortical plate, so that the positional information of their origin is preserved. Radial glia cells play a prominent role in the enormous cortical expansion during evolution, since the size of the cortical sheet depends on the number of contributing radial units, while the thickness of the cortex depends on the magnitude of cell production for each unit. The initial number of radial units in a given species is likely to be genetically determined in the early stages of embryogenesis, while the organization and final size of

the cytoarchitectonic areas are refined once connectivity with other structures and areas is established.

The pioneer observations of post-mitotic neurons aligned with radial glial fibers during the development of cerebellar and cerebral cortices (Rakic, 1972) have been extensively supported by many *in vitro* (Anton et al., 1996) and *in vivo* studies (Noctor et al., 2001). In agreement with this model different molecular abnormalities affecting radial glia cells lead to abnormal neuronal migration (Ross and Walsh, 2001).

Neuronal radial migration is thought to involve at least two different modalities of cellular movement. As described above the first cohort of neurons that migrate out of the cortical VZ constitutes the so-called preplate. A successive wave of neuronal migration gives rise to the cortical plate, which splits the preplate into two layers, the marginal zone and the subplate. The following waves of neuronal radial migration position neurons in different layers of the cortical plate (future cortical layers 2-6).

The neurons destined for the cortical plate use the radial glia fibers to reach their final position. Cells that adopt this glia-guided locomotion have a short leading process that is not attached to the pial surface and display a saltatory pattern of locomotion (Nadarajah et al., 2001).

A second type of radial migration has been described for cortical cells migrating out of the VZ at early stages of corticogenesis. In this type of radial migration (soma translocation) translocating cells first extend a process to the pial surface as they leave the VZ, and then they lose their ventricular attachments while maintaining their pial connections (Nadarajah et al., 2001).

Thus, the migratory behaviour of translocating cells is independent and distinct from those undergoing glial-guided locomotion, and their migration is relatively continuous with the leading processes becoming progressively shorter. Therefore, the molecular mechanisms of migration involved in somal translocation and glia-guided locomotion appear to be

different. Accordingly, mutations affecting the cascade of signalling mechanisms that regulate glia-guided migration do not severely affect the formation of the pre-plate (Nadarajah et al., 2001), whereas defects of the pial basement membrane (which is required for both types of migration) affects both the development of the preplate and cortical plate (Graus-Porta et al., 2001; Halfter et al., 2002).

The migration of post-mitotic neurons to the cortical plate from the VZ involves at least four consecutive and partially overlapping processes: (i) initiation of movement, (ii) attachment to the radial glia fiber, (iii) locomotion, which involves nucleokinesis, and (iv) detachment of the radial glia and acquisition of appropriate laminar position.

(i) Modulation of radial movement by motogenic factors

Brain-derived neurotrophic factor (BDNF) and NT-4, members of the neurotrophin family, have been shown to promote the migration of cortical neurons. Their receptor (TrkB) is expressed in migrating neurons in the cortical plate (Behar et al., 1997), and direct infusion of NT-4 or BDNF into the lateral ventricle augments neuronal migration and formation of heterotopias (Brunstrom et al., 1997). Neurotransmitters also play a role in modulating the migration of cortical projections neurons: $GABA_{a/c}$ receptors are involved in the cell movement from the proliferative zone to IZ, whereas $GABA_b$ receptors appear to influence migration from the IZ to the CP (Behar et al., 2000). Furthermore, blockade of NMDA receptors decreases cell migration (Behar et al., 1999).

(ii) Neuronal-glial interactions

Several molecules have been implicated in regulating the interaction of migrating cells with the radial glia, such as Astrotactin-1 (Astn1), and -2 (Astn2) (two glycoproteins expressed by migrating neurons), and integrins (cell-surface glycoproteins that mediate cell-cell and cell-extracellular matrix interactions).

(iii) Locomotion during radial migration

A critical aspect during radial glial migration concerns adaptation of the microtubule network, which is responsible for the extension of a leading process and the translocation of the nucleus. Several genes are involved in the regulation of cytoskeleton rearrangement during radial migration, and among them Lis1 (non catalytic β1 sub-unit of the platelet activating factor acetyl-hydrolase) encodes for a protein involved in multiple protein-protein interactions. Mutations in human Lis1 cause a severe form of Lissencephaly. Doublecortin, another gene whose mutation in human leads to X-linked Lissencephaly (Gleeson et al., 1998), encodes for a microtubule-associated protein critical for the stabilization of the microtubule network. Mutations in the actin-binding protein Filamin α (FLNA) cause periventricular heterotopia, a migration disorder in which neurons carrying the mutation accumulate close to the progenitor zone of the cerebral cortex

(iv) Layer formation

Once migrating neurons reach their final destination, they detach from the radial glia processes and halt their migration. Neuronal layers in the cortex are established according to an inside-outside pattern, and interaction between migrating neurons and Cajal-Retzius cells is essential for this process. After the first "late-born" neurons (future layer 6) split the preplate into the marginal zone and the subplate, successive waves of neurons pass over previous ones until they reach the marginal zone, where they detach from the radial glia. The Cajal-Retzius cells of the MZ secrete Reelin, a large protein responsible for correct layering, and mutation of reelin causes defects of neuronal migration typical of the reeler mice (D'Arcangelo et al., 1995) and Lissencephaly with cerebellar hypoplasia in humans if it is mutated (Hong et al., 2000). In *reeler* mice, the first migrating cells fail to split the preplate, thus the following waves of neurons stack up and accumulate below the subplate (called the superplate in reeler mutant) and below cells that migrate

previosly, creating a cortex with inverted layers. Reelin is a high affinity ligand for two members of the LDL family of lipoprotein receptors, the very-low-density lipoprotein receptor (VLDLR) and the low-density lipoprotein receptor related protein 8 (ApoER2), which are both expressed by the migrating cortical cells (D'Arcangelo et al., 1999). The two receptors mediate Reelin internalisation and tyrosine phosphorylation of DAB1 (D'Arcangelo et al., 1999; Howell et al., 2000), a cytoplasmic adaptor protein interacting with the cytoplasmic tail of VLDLR and ApoER2 and linked to reorganization of the cytoskeleton. A second pathway controlling neuronal positioning involves the cyclin-dependent kinase 5 (Cdk5) and its activating subunits p35 and p39. Defects in Cdk5 signaling do not affect normal splitting of the preplate but still cause an inversion of cortical lamination similar to *reeler* mutants. These findings suggest that the splitting of the preplate and the acquisition of normal lamination are probably two partially independent processes. It has been suggested that Reelin could act as a stop signal for radially migrating neurons (Dulabon et al., 2000), or alternatively it could directly regulate the identity and function of radial glia (Super et al., 2000), or it could simply promote the detachment of migrating neurons from the radial glia processes. Successive waves of neurons born from the same radial progenitor cell ordinarily use the same radial processes to reach the cortical plate, and the lack of detachment of earlier neurons may constitute a physical barrier for the migration of subsequent neurons toward the MZ.

Tangential migration

It has long been recognized that cells migrate and distribute in the forebrain in patterns that do not coincide with the arrangement of the glial fibers system. In the "so-called" tangential migration cells use different substrates to promote migration: they can use each

other to support their migration, they can follow growing axons, and finally they may not follow specific cellular substrates.

In mammals, precursors of olfactory interneurons (periglomerular and granular cells) are born in the subpallium and reach their destination in the OB through a tangential migration (Altman, 1969; Lois and Alvarez-Buylla, 1994). Several lines of evidence (Wichterle et al., 2001) (Corbin et al., 2000; Sussel et al., 1999) suggest that precursors of olfactory interneurons are mainly generated in the dorsal region of the lateral ganglionic eminence (LGE), from where they migrate throughout adulthood (Altman, 1969; Lois and Alvarez-Buylla, 1994), providing a constant neuronal supply for the continuous remodelling of GABAergic circuits in the olfactory bulbs.

The origin of olfactory interneuron precursors in the postnatal telencephalon is the subventricular zone (or better subependymal zone) (Altman, 1969; Lois and Alvarez-Buylla, 1994), internal to the ependymal layer and surrounding most of the lateral ventricles and which is thought to derive from the LGE. The migration of the olfactory precursors in the adult occurs along a specific pathway termed *rostral migratory stream* (RMS) (Kornack and Rakic, 2001; Lois and Alvarez-Buylla, 1994), in which chains of migrating interneurons are ensheathed by astrocytes (Peretto et al., 1997).

Several molecules are involved in this process, including:

- the polysialylated form of the neural cell adhesion molecule (PSA-N-CAM),
- Tenascin-C (a ligand for αvβ3 and åvβ6 integrins (Yokosaki et al., 1996)), which is also strongly expressed in the astrocytes encasing the cellular chains,
- proteins that mediate cell-cell contact such as the Eph family of tyrosin kinase and their membrane associated ephrin ligands.

Migration of interneuron precursors from the sub-pallial telencephalon to the olfactory bulb is a highly directional process (Lois and Alvarez-Buylla, 1994), in which the bipolar migrating cells have short trailing process tipped by large growth cones oriented toward

the olfactory bulb (Murase and Horwitz, 2002). In the embryonic brain a different set up has been proposed for directing migration to the OB, in which chemorepulsive factors present in the septum direct migration of the immature interneurons (Hu and Rutishauser, 1996). Slit1 and Slit2, two secreted proteins that interacts with Robo receptors (Brose et al., 1999; Li et al., 1999), have been identified as the candidate molecules for this activity. The olfactory bulbs of Slit1 and Slit2 double mutant are, in fact, smaller than normal at birth (Marin O, unpublished results). In addition to a repulsive activity, it has been also suggested that an attractive activity present in the olfactory bulb contributes to direct the migration. For instance, Netrin1 is expressed in mitral cells of the olfactory bulb, whereas the Netrin1 receptors DCC and Neogenin are expressed in migrating cells from E15 to P5 (Murase and Horwitz, 2002). Nevertheless, this hypothesis is not in agreement with the reduction of Netrin1 expression after P4, and with the finding that the RMS and migration of SVZ precursors persists after ablation of the olfactory bulb (Jankovski et al., 1998). Accordingly, it has been suggested that migration in the adult RMS seems due to a combination of Slit-mediated repulsion from the SVZ and intrinsic motogenic activity in the RMS (Mason et al., 2001).

The embryonic subpallium is the origin of a large number of cells that migrate tangentially to the developing cerebral cortex and hippocampus (Anderson et al., 1997b; de Carlos et al., 1996; Lavdas et al., 1999; Letinic et al., 2002; Pleasure et al., 2000; Wichterle et al., 1999). These cells give rise primarily to GABAergic interneurons (Anderson et al., 2002; Cobos et al., 2001), although there is evidence that the subpallium generates also cortical oligodendrocytes during embryogenesis (Olivier et al., 2001). Cells tangentially migrating to the cortex seem to have multiple origins within the subpallium (Anderson et al., 2001; Nery et al., 2001), although the vast majority of GABAergic interneurons derive from the MGE (Sussel et al., 1999; Wichterle et al., 1999; Wichterle et al., 2001), and the oligodendrocytes from the entopeduncular area (AEP) (Olivier et al., 2001; Spassky et al.,

1998). The MGE is also the source of interneurons for other forebrain structure, such as the striatum (Marin et al., 2000).

Interneurons migrating to the cortex follow two restricted routes. They either (i) travel as compact clusters along the ventricle in the subpallium and once they reach the cortex enter the IZ (midgestation) or the SVZ (late gestation); or (ii) they move from the proliferative zone to the superficial mantle layer of the subpallium and then migrate through the marginal or subplate zones of the cortex (Lavdas et al., 1999).

Three different elements are required for the tangential migration: (i) factors that promote cellular mobilization, (ii) substrate molecules, and (iii) molecular cues directing the interneurons to their targets.

(i)-motogenic factors

Tangentially migrating neurons have an outstanding migratory capability (Wichterle et al., 1999), which suggests that they are highly responsive to scatter factors in the telencephalon. One of these molecules is the hepatocyte growth factor (HGF), which is expressed in the telencephalon at the time when interneurons migrate to the cortex (Powell et al., 2001). Tangential migration of interneurons is also strongly stimulated in vitro by BDNF and NT4 and attenuated by tyrosine kinase inhibitors (Polleux et al., 2002).

(ii) substrate molecules

Presently, these factors are still unknown. Tangential migration appears to be independent of interactions with radial glia cells. Nevertheless, some tangentially migrating cells in the cortical IZ appear to be closely associated with corticofugal axons (Denaxa et al., 2001; Metin et al., 2000). Antibodies against TAG1, which is expressed on corticofugal axons, reduce the number of interneurons reaching the cortex in slice cultures (Denaxa et al., 2001). However, analysis of Tag1 mutant mice has not revealed major alterations in tangential migration (Denaxa et al.,

2003). Moreover, the fact that tangentially migrating cells in the lower IZ and SVZ avoid the axon rich upper part of the IZ, suggests that these cells preferentially use other substrates than axons

(iii) directional guidance of migrating interneurons

The ventral to dorsal interneuron migration appears to be established and guided by the simultaneous activity of chemorepulsive and chemo attractive factors produced by the preoptic area (POa) and the cortex, respectively (Marin and Rubenstein, 2003; Wichterle et al., 2003). A repulsive activity in the preoptic area prevents interneurons from migrating ventrally and facilitates their dorsal migration toward the cortex. It is of interest to note that this activity is still present even in mice with null-mutations of both Slit1 and Slit2 genes, which encode for chemorepulsive factor in the ventral telencephalon (Marin and Rubenstein, 2003). The existence of a diffusible cortical attractive activity (CAA) for tangentially migrating interneurons has been recently revealed by two independent studies (Marin and Rubenstein, 2003; Wichterle et al., 2003). In matrigel matrix experiments, MGE cells preferentially migrate toward cortical cells, and in slice cultures, the addition of an ectopic cortex deviates the migration of MGE-derived cells (Marin and Rubenstein, 2003). A second study demonstrated that the genetic disruption of the embryonic cortex in Emx1/Emx2 double mutants reduces the tangential migration from the subpallium (Shinozaki et al., 2002). Furthermore, migration of interneurons from the MGE to the subpallial/pallial boundary is largely independent from the cortex (Marin et al., 2003), indicating that the role of the CAA may be to guide interneurons once they reach the pallium.

Guidance cues are also required to properly distribute interneurons to the different telencephalic structures. For example, sorting the interneurons fated to the cerebral cortex or striatum appears to be mediated by Neuropilin/Semaphorin interactions

(Marin et al., 2001). Neuropilins are transmembrane receptors that mediate the axonal repulsive actions of class 3 semaphorins (Raper, 2000), and in the subpallium, Neuropilin1 and Neuropilin2 are co-expressed in the interneurons migrating to the cortex and not in those fated to the developing striatum. Expression of neuropilins seems to prime the migrating cortical interneurons to respond to the striatal chemorepulsive activity where class 3 semaphorin are expressed, and conversely loss of Neuropilins function increases the number of interneurons migrating to the striatum. The guidance of tangentially migrating neurons may also be influenced by neuronal activity. In fact there is evidence that glutamate release from corticofugal axons leads to receptor activation in tangentially migrating cells and modulates their response to guidance cues.

Once they reach the pallium, interneurons invade the cortex and distribute into the different cortical layers, and it has been shown that cortical interneurons, like projection neurons, are generally positioned in an inside-outside order in the cortical layers with respect to their birthdates. Moreover, interneuron progenitors born at a particular stage are not irreversibly committed to specific layers, but they can be respecified by the host environment, displaying pluripotent characteristics with respect to layer fates (Valcanis and Tan, 2003). It is interesting that molecules that influence migration of projection neurons do not seem to influence the tangential migration of interneurons, except for small subpopulation of GABAergic neurons that are altered in *reeler* mutant cortex (Renfro A, Rubenstein, D'Arcangelo unpublished). Interestingly, cortical interneurons appear to seek the cortical ventricular zone before moving radially to reach their position in the cortical plate (Nadarajah et al., 2002), indicating their final positional information may be instructed in the ventricular zone.

Most forebrain structures (cortex, hippocampus, olfactory bulb, striatum, and hypothalamus) arise from the integration of neurons arriving via radial and tangential migration, indicating that probably patterning and neuronal migration are intimately linked during CNS development. The generation of specific populations of telencephalic neurons with distinct neurotransmitter phenotypes appears to correlate with the original location of the progenitor cells along the dorso-ventral subdivisions of the telencephalon, and tangential migration might be a mechanism selected through evolution to increase the cellular complexity of specific circuits. The discovery that correct forebrain development requires radial and tangential migrations has important clinical implications. Because the majority of the neurons in the cortex are projection neurons, disruption of the migration of these neurons typically results in severe malformations of the cortex (Ross and Walsh, 2001). In contrast, defects in the migration or final arrangement of cortical interneurons may lead to more subtle morphological defects that nevertheless may cause severe impairment of cortical function.

Role of transcription factors in forebrain development

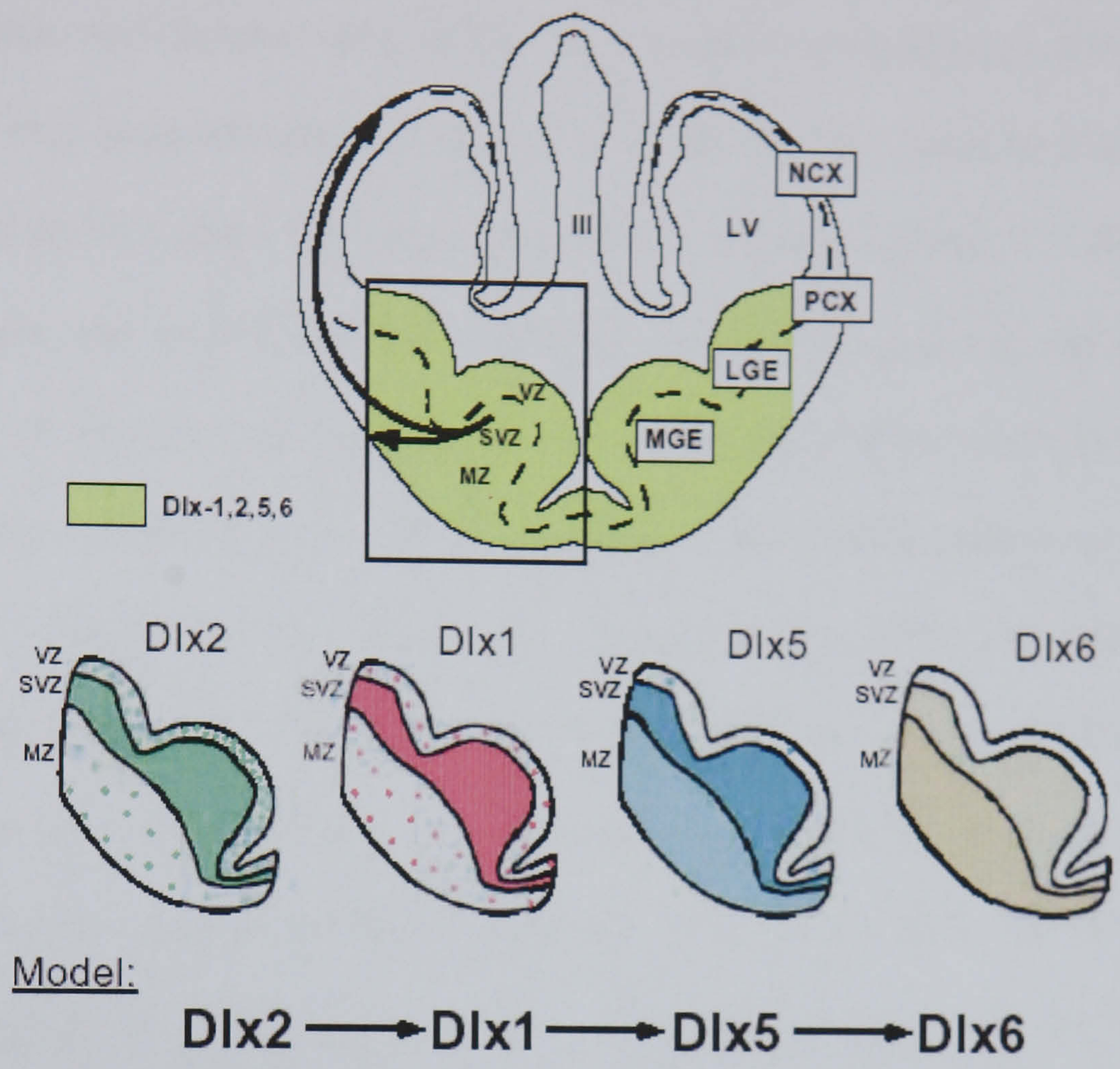
Forebrain development is the result of a fine-tuned regulation of differential proliferation of the progenitor cells, cellular specification, differentiation and migration, and many transcription factors seem to be involved in the regulation of these processes. Many of these genes are also expressed in regionally restricted patterns with boundaries corresponding to the limits of histogenetic primordia of the different telencephalic structures.

Dlx genes

This family of homeobox genes is characterized by expression specifically restricted to the anlage of the basal ganglia. Outside the brain these genes are expressed in the branchial

arches, limbs bud, and enteric nervous system. In the developing basal ganglia Dlx1 and Dlx2 genes are expressed largely in the proliferating cells of the ventricular and subventricular zones (Bulfone et al., 1993; Porteus et al., 1994), whereas Dlx5 and Dlx6 are expressed in progressively more differentiated cells, suggesting that this gene family regulates different stages of differentiation in the forebrain. In mice and humans there are six known Dlx genes, which are found in three convergently transcribed pairs (Dlx1 and 2, Dlx 3 and 4, Dlx 5 and 6). The intergenic regions of each pair contain some of the enhancer elements, and pairs of the murine Dlx genes exhibit similar patterns of expression that are generally conserved in their non-mammalian counterparts. Within the neural tube, the expression of Dlx1, Dlx2, Dlx5 and Dlx6 is highly restricted to two forebrain domains (one diencephalic and one telencephalic), where the expression follows the temporal sequence: Dlx2, Dlx1 and Dlx5, then Dlx6 (see Fig 5).

Fig. 5



Expression domain of Dlx1, Dlx2, Dlx5 and Dlx6 during mouse brain development in a transverse section thorough the E12.5 mouse telencephalon. The arrows indicate the migration from the subpallium to the pallium. (Bottom) A hypothesized genetic and biochemical pathway that proposes the sequential role of Dlx genes at different stages of differentiation.

Abbreviations: NCX: neocortex; PCX: palliocortex (pallial territories). LGE: lateral ganglionic eminence; MGE: medial ganglionic eminences (subpallial territories)

Dlx2 is individually expressed in subsets of ventricular zone neuroepithelial cells. Dlx1, Dlx2 and Dlx5 are expressed together in most subventricular zone cells, and while Dlx5 and Dlx6 are expressed in most of the post mitotic differentiating neurons. Dlx2 and Dlx1 are expressed in a restricted subset of postmitotic neurons. This temporal and regulatory cascade among the Dlx genes has been confirmed by the analysis of the Dlx1/Dlx2 double mutants, which also proved that the expression of the Dlx genes is critical for the development of virtually all neurons that use γ-amino butyric acid (GABA) as a neurotransmitter (Anderson et al., 1997a; Anderson et al., 1997b; Stuhmer et al., 2002a; Stuhmer et al., 2002b). In fact, ectopic expression of Dlx2 or Dlx5 in cortical neurons induces expression of the GABAergic phenotype (Stuhmer et al., 2002a), and the Dlx1/Dlx2 double mutants exhibit a major block in GABAergic neurogenesis in the subcortical telencephalon (Anderson et al., 1997b; Marin et al., 2000). In the Dlx1/Dlx2 double mutants, the first wave of neurogenesis (from approximately E10-E12) appears to be undisturbed, whereas differentiation of later born neurons is largely aborted. This leads to abnormalities in the SVZ, the region that contains the secondary proliferative population (spp) of neuroblasts, while the primary proliferative population (ppp) appears normal. The mutant spp expreses Lhx2, Notch1 and its ligand Delta1 (Porter et al., 1997), which are all characteristic of the normal ppp. An increase in notch signalling is in agreement with the block of differentiation in the Dlx1/Dlx2 double mutants, that is reflected by the increased expression of Hes5, the lack of Dlx5/Dlx6 expression (spp markers), and a block in the radial migration of these cells to the postmitotic zone (mantle) (Anderson et al., 1997b). This block in differentiation not only reduces the production of basal ganglia late-born projection neurons (GABAergic neurons that project to distant targets), but it also impairs the development of GABAergic, dopaminergic and cholinergic interneurons (Anderson et al., 2001; Anderson et al., 1997b; Marin et al., 2000). In the Dlx1/Dlx2 double mutant there is a massive reduction in the GABAergic interneurons of the cerebral cortex

(Anderson et al., 1997a; Anderson et al., 2001; Marin and Rubenstein, 2001) due to the lack of tangentially migrating immature interneurons from the subcortical telencephalon. Thus, a correct Dlx function is critical for the development of the neurons that originate from the basal telencephalon and producing GABA, acetylcholine and dopamine. It is conceivable to hypothesize (Rubenstein and Merzenich, 2003) that a reduced number or function of cortical inhibitory GABAergic neurons could be responsible for hyperactivity states, such as seizures, or result in the disruption of local cortical circuits. Likewise, GABAergic dysfunction in the basal ganglia could interfere with a correct development of complex motor and cognitive behaviours.

bHLH genes

There are morphological, physiological and molecular characteristics shared by all types of neurons. Despite the similarities, however, neurons constitute the most diverse cell population of the organism. Recently, big efforts have been focused on the identification of the molecular mechanisms underling this cellular diversity.

Genetic studies in Drosophila and vertebrate models have provided evidence that a small number of so called "proneural genes", encoding transcription factors of the basic helix-loop-helix (bHLH) family (Bertrand et al., 2002), are both necessary and sufficient to generate progenitors committed to the neuronal lineage and have a critical role in cell fate specification during corticogenesis.

The proneural bHLH genes: Mash1, Ngn1 and Ngn2

In the telencephalon, Mash1 is expressed at high levels in the subpallial progenitors, whereas Neurogenin1 (Ngn1) and Neurogenin2 (Ngn2) are exclusively expressed in the pallial progenitor cells. This restricted pattern of expression reflects a distinct role for these genes in the specification of the basal ganglia and cortical neuronal identities. It has been shown that Ngn expression is required to prevent the spreading of ectopic Mash1

expression in the dorsal telencephalon, thus ensuring that the pallial and subpallial subdivisions of the telencephalon express different sets of neuronal determination genes (Fode et al., 2000). The importance of keeping the Mash1 expression restricted to the ventral telencephalon is highlighted by the observation that ectopic Mash1 expression in Ngn mutants results in the mis-specification of many early born cortical neurons, which lose expression of several dorsal specific markers (e.g. Tbr1, Math2) and conversely express the ventral markers Dlx1, Dlx2, Dlx5 and GAD67. The role of Mash1 is confirmed by the fact that the forced expression of Mash1 in dorsal telencephalic progenitors is sufficient to confer ventral characteristics to the cortical neuronal progeny (Fode et al., 2000), indicating unequivocally that Mash1 and Ngn1/Ngn2 are involved in neuronal specification and acquisition of specific regional neuronal phenotypes during forebrain development.

The "differentiating" bHLH genes: NeuroD, NeuroD2 and Nex (Math2)

Over expression of Mash1 (like Ngn1) induces the expression of panneuronal markers, such as β-tubulin, indicating that these factors can initiate the program of gene expression specifically instructing the neuronal lineage (Farah et al., 2000). However, it is known that a cascade of bHLH gene expression is involved in the specification and differentiation of neuronal cells (Ross et al., 2003), while the proneural bHLH factors are involved in the specification of the progenitors to a neural (neural and glial) fate. The NeuroD/Nex gene family, which include NeuroD, NeuroD2, and Nex (also called Math2), act to terminally differentiate the neural committed cells into neurons (Lee, 1997). The expression of these bHLH differentiation genes begins in the immature neurons of the cortical plate and is maintained during neuronal differentiation (Lee et al., 2000).

Inhibitors of bHLH factors: Hes1 and Hes5

The regulation of cortical cell number is a complex developmental task that the telencephalon is able to perform by a fine tuned control of cortical progenitor proliferation versus differentiation. Two families of inhibitory bHLH proteins, the Hes and Id factors, are directly involved in this process, and their role is to antagonize the neurogenetic proneural bHLH factors, and to promote cell cycle progression (Id genes). Hes1 and Hes5 are expressed in the telencephalic ventricular zone (Allen and Lobe, 1999) where they maintain the progenitors in an undifferentiated, proliferative state and inhibit their differentiation into neurons (Nakamura et al., 2000; Ohtsuka et al., 2001). In fact, in the null mutant Hes1 mice there is a premature neuronal differentiation, with a 2-fold excess of forebrain neurons at E13.5 and a reduced final number of cortical neurons at birth (Nakamura et al., 2000). Several studies have revealed that Hes proteins inhibit neuronal differentiation through two distinct mechanisms: (i) Hes factors form homodimers and heterodimers with closely related family members and bind to DNA elements called N boxes (CACNAG) to repress the expression of target genes, such as the proneural gene Mash1, that are required for neuronal differentiation (Davis and Turner, 2001) (transcriptional repression is mediated by the interaction of Hes proteins with transcriptional co repressors of the Groucho/transducin-like enhancer of split (Gro/TLE) family); (ii) Hes factors interact physically with proneural bHLH proteins antagonizing their activity. Hes1 and Hes5 are also key target genes that are transactivated in response to notch signalling (Justice and Jan, 2002), and in the cortical progenitors of Hes1/Hes5 double mutants, constitutively active notch signalling is unable to block neurogenesis (Ohtsuka et al., 1999). Hes1 and Hes5 are, in fact, Notch effectors that specifically promote the maintenance of the cortical progenitor compartment by blocking their differentiation. Newly formed neurons up-regulate the Notch ligand, Delta, as they differentiate, and Delta activates Notch/Hes signalling in adjacent progenitor cells.

blocking differentiation. Thus lateral inhibition allows the selective differentiation of some cortical progenitors into neurons while preventing simultaneous neurogenesis of all cortical progenitors.

Pax6

Pax6 is a highly conserved member of a family of transcription factors containing the paired and homeobox DNA-binding domains, which plays a crucial role in the development of the vertebrate CNS. The mouse *Small eye* (allele *Sey*) mutation is caused by a point mutation in the Pax6 gene, resulting in the production of a non-functional protein, and the homozygous *Small eye* (Sey/Sey) animal dies at birth with multiple CNS defects (eye, forebrain, cerebellum, and spinal cord) (Simpson and Price, 2002). In the embryonic telencephalon, the expression of Pax6 is confined to the mitotically active ventricular neuroepithelium of the pallium, and the development of the cortex is severely affected in the *Sey/Sey* mutant: the CP is hypocellular without radial glia alignment of the cells, and the VZ/SVZ/IZ are engulfed by precursors in large clumps (Warren et al., 1999), which highly express the mutant Pax6 message (Stoykova et al., 1997; Stoykova et al., 2000) and actively incorporate BrdU after pulse labelling throughout development (Brunjes et al., 1998; Warren et al., 1999). In the mutant telencephalon there is also a severe defect in DV patterning (Stoykova et al., 2000), with a dorsal shift of the pallial/subpallial border in the VZ-SVZ, resulting in a ventralized and malformed basolateral cortex. Moreover the proliferative VZ-SVZ of the VP and LP in Sey/Sey embryos expresses ectopically Dlx1, Mash1, Vax1 and Six3, whereas the restricted expression of Nkx2.1 and Lhx6 to the MGE expands into the adjacent LGE territory. From E14.5 onward, the pallium of the Sey/Sey mutant fails to properly differentiate, and cells accumulated in the mutant VZ-SVZ express the neuron specific marker TuJ1 (Caric et al., 1997), but not the differentiation markers Tbr1 and Emx1, suggesting that a portion of the late cortical progenitors are either not generated or are unable to properly differentiate

(Stoykova et al., 2000). Pax6 has also an essential role in the differentiation of the cortical RC2+ radial glial cells (Gotz et al., 1998), and isolated radial glia cells from Sey Sey cortex are able to generate neuronal clones at a much lower rate if compared to the WT radial glial cells (Malatesta et al., 2000).

Nkx2.1

Nkx2.1 is a member of the vertebrate Nkx family (Pera and Kessel, 1998; Qiu et al., 1998), and it is also known as TTF1 or T/ebp because of its ability to regulate the expression of thyroid specific gene products (Guazzi et al., 1990). The early expression of Nkx genes is induced by SHH and is restricted to the medial area of the neural plate, overlying the SHH-secreting axial mesendoderm. Within the medial neural plate and ventral neural tube, the expression domains of the Nkx genes have a distinct anteroposterior and dorsoventral organisation (Qiu et al., 1998). For instance, while Nkx2.2 and Nkx2.9 are expressed along the entire CNS in a narrow column of cells adjacent to the floor plate (Shimamura et al., 1995), Nkx2.1, which is one of the earliest known genes to be expressed in the forebrain, is expressed in the hypothalamic primordium already at one somite stage (Shimamura et al., 1995), and by 11 somite stage in the entire rostrobasal telencephalon, a region that develops into the preoptic area, anterior entopeduncular area, medial ganglionic eminence, septum, and parts of the amygdala. In these regions Nkx2.1 is expressed in both progenitor and postmitotic cells, indicating its involvement in precursor specification and differentiation of mature ventral forebrain cells. In Nkx2.1 null-mutants the MGE appears to be respecified to the fate of the more dorsal LGE structure (Sussel et al., 1999), and it does not give rise to its normal derivatives (e.g. the globus pallidus), but to the striatum, the LGE derivative. Moreover, the lack of a normal MGE results in an absent cell migration from the pallidum into the striatum (cholinergic neurons) and into the cortex (GABAergic neurons and calbindin-positive cells) (Sussel et al., 1999). Thus, Nkx2.1 is

required both for regional specification of the ventral telencephalon and for the production of specific cell types that migrate into the striatum and cerebral cortex.

Genetic interactions

Although the telencephalic subdivisions were initially delineated on the basis of differences in morphology, connectivity and neurochemical profiles, dorsal and ventral domains of the telencephalon are also distinguished embryonically by distinct patterns of gene expression, reflecting the initial acquisition of regional identity by progenitor populations. Regionally restricted gene expression participates in the specification of the identity of the telencephalic territories and a genetic code underlying the specification of progenitor identity in the telencephalon can be hypothesized. For instance, Pax6 operates upstream of Ngn2 (probably in a direct manner) (Scardigli et al., 2003), but several genes, probably upstream of Pax6, including Emx1 and Emx2, are unaffected in both Ngn2 and Pax6 mutants, and the molecule upstream of the Emx genes is the Gli3 zinc finger transcription factor (Theil et al., 1999).

Cerebral cortical area map

The mammalian cerebral cortex is a continuous sheet of neural tissue with complex regional differences: the different types of cortex range from the six-cell-layered neocortex to the one cell layered archicortex. Within these large subdivisions, distinct areas have specialized functions, which form a map that is similar from one individual to another and has common topological features across mammalian species.

The process of arealization involves the subdivision of the cerebral cortex into anatomically and functionally distinct areas, which altogether form a species-specific area map. Neural structures are patterned in the embryo by signaling centers that release signaling proteins that regulate regional growth and specify the regional identity in the tissue. These proteins, named morphogens, diffuse in the tissue in gradients that directly

confers positional information. In well-studied model systems, such as the *Drosophila* embryo or the embryonic vertebrate spinal cord, cells respond to different levels of morphogens by expressing specific transcription factors (Wolpert, 1996), which in turn induce regional expression of downstream genes that regulate locally the final differentiation of the tissue (Briscoe and Ericson, 2001). The development of structurally and functionally specific cortical area map, however, cannot be explained only by the role of patterning morphogens: a key feature of the cortical arealization is that different areas receive distinct sets of projections from different thalamic nuclei, which act as relay centers for information coming from the periphery and other parts of the brain. Two classical models of cortical development have dominated in these past years: (O'Leary, 1989; Rakic, 1988) (i) the *protomap* model: the cortical primordium is patterned as it is generated, and the intrinsic area differences are specified by molecular determinants active in the ventricular zone (VZ). As newborn neurons migrate out of the VZ in radial arrays they carry the areal protomap information with them to the cortical plate. This was most convincingly verified by the analysis of the Gbx2-/- mouse where thalamic afferents fail to reach the neocortex, because of a thalamic defect (Miyashita-Lin et al., 1999), and the expression of various areal markers in the neocortex remains relatively unchanged, showing that intrinsic cortical patterning alone is sufficient to determine the molecular arealization. (ii) The *protocortex* model: some early embryonic cortical transplantation experiments suggested a prolonged plasticity in conferring areal identity (O'Leary, 1989; Schlaggar and O'Leary, 1991) and supported the hypothesis that the cortical VZ is essentially homogeneous as it is generated, and is specified into areas by patterning cues from growing thalamic axons.

It is possible to draw a new protomap model of cortical patterning, based on current knowledge of other embryonic systems (*Drosophila*, for example), in which signaling molecules released from discrete centers provide early positional information and exert

regional growth control. These signals set up early cortical domains characterized by the expression of specific transcription factors, which then control regional specification and growth. According to this model the disruption of the signaling centers or transcription factors will disrupt the cortical map. Given that in other systems basic patterning occurs before major growth, this model also predicts that the cortex shows regional differences early in corticogenesis, and because afferent connections arrive relatively late in development, the cortical areal pattern should emerge both independent of, and earlier than, extrinsic innervation.

Evidence for early cortical patterning prompts a search for the cortical signaling centers. A prominent feature of the cortical map is that many areas are arranged in roughly longitudinal bands along the medial and lateral edges of the cortical sheet, and this organization suggests that centers may lie along the edges of the embryonic cortex, providing positional cues along the medial-lateral axis. Other signaling centers might be positioned to regulate the anterior-posterior axis, and consistent with this possibility, sources of BMP, WNT, EGF, FGF, and HH signals, have been identified at the appropriate location at or near the boundaries of the embryonic cortex.

Adult neural stem cells

Defining neural stem cells

A stem cell is defined as a cell capable of extensive proliferation and able to give rise to other stem cells (self-renewal) as well as to progeny that will terminally differentiate and integrate in the tissue of residence.

The stem cell compartment has a critical role in development and in the maintenance of specific tissues in most animals. Current research is focusing on the identification of the characteristics and on the potential application of neural stem cells (NSC) in both developing and adult system.

Self-renewal and proliferation

A cardinal feature of stem cells is their ability to self-renew, namely to give rise to at least one daughter cell that maintains the exact *multipotent* character of its parent (Morrison et al., 1997). Stem cells can divide symmetrically during development to expand their number, and asymmetrically to self-renew and give rise to a differentiated progeny. The mechanisms underlying mitotic asymmetry in neural stem cells are of great interest; studies in *Drosophila* have identified a number of genes whose mRNA and/or protein are asymmetrically localized in progenitor cells prior to division (Lu et al., 2000), such as NUMB, and can interact with genes that influence cell fate by mediating cell-cell interaction, such as NOTCH. In the analysis of cortical progenitor cell lineage trees show that the asymmetric cell division is similar to the *C. Elegans* or *Drosophila* neural progenitor lineages (Shen et al., 1998). Progenitor cells have been isolated recently from the embryonic mouse cortex and a significant association was found between asymmetric Numb distribution and asymmetric cell fate as they undergo cell division (Shen et al., 2002). As a matter of fact, it has been demonstrated that at E13-E14, at the peack of asymmetric neurogenesis, Numb moves preferentially into the committed neuronal daughter cell.

In the mature nervous system the assumption was that neurons were incapable of cell renewal, but studies carried out in the last ten years have uncovered an unexpected postnatal, and adult neurogenetic compartment in mammalian CNS. The confirmation that neurogenesis occurs in at least some areas of the adult brain has attracted attention to the possibility of inducing and mobilizing such endogenous stem cell compartments as an alternative strategy for the therapy of brain diseases. Nevertheless, it has been considered that BrdU or [^{3}H] thymidine incorporation, commonly used as criteria of neurogenesis, are not markers of cell division but indicators of DNA synthesis, and damaged or degenerating neurons can activate cyclins and initiate abortive DNA synthesis without mitosis (Rakic,

2002). Thus in the absence of definitive markers that can be correlated with the key functional properties of multipotency and self-renewal, stem cells cannot be positively identified *in situ*.

Developmental potential

A stem cell gives rise to committed progeny that will terminally differentiate and integrate into the tissue of residence, and in the central nervous system will generate neurons, astrocytes, and oligodendrocytes. A number of investigators have demonstrated that neural stem or progenitor cells expanded *in vitro* and transplanted into different brain regions have a remarkably broad developmental capacity. Moreover, it has been shown that stem cells derived from a tissue can give rise to differentiated cell types typical of other organs, to which they do not normally contribute (Morrison, 2001). For example, neural stem cells are able to generate blood cells (Bjornson et al., 1999) and skeletal muscle (Galli et al., 2000) and they could contribute to multiple tissues from all three germ layers if transplanted into host embryos at early stages (Clarke et al., 2000). In these experiments NSCs were generally isolated by selective growth in defined media containing FGF2 and/or EGF, and such *ex vivo* expansion may broaden the developmental potential of the cells by reprogramming them (Palmer et al., 1999). The term "reprogramming" should imply the acquisition (or reacquisition) of differentiation capabilities that stem cells do not normally possess at the time of isolation. The molecular basis of this process is unclear, but may involve large-scale chromatin reorganization, demethylation, or exposure to epigenetic changes that are not usually required for competent progenitors to generate their normal repertoire of differentiated cells.

Neurogenetic compartments

Neurogenetic niches of the adult mammalian brain:

- The external granular layer of the cerebellum (EGL).
- The olfactory neuroepithelium (ON).
- The olfactory bulbs (OB).
- The hippocampus (EGL).

The external granular layer of the cerebellum persists as an actively mitotic layer generating granule cells for the internal granular layer through the first three postnatal weeks in rodents. The EGL of the cerebellum originates after a massive tangential cell migration from the wall of the fourth ventricle, followed by a centripetal migration of neuroblasts along radial glia cells.

The olfactory neuroepithelium consists of only one neuronal cell type, the olfactory receptor neurons, which are continuously renewed through differentiation of *unipotent* basal neural stem cells (they can give rise to only one cell lineage) (Calof et al., 1998).

The olfactory bulb (OB) is characterized by a continuous cell renewal both in its main (related to olfaction) and accessory (related to pheromones) parts (Bonfanti et al., 1997). The region where the cells actively proliferate is the remnant of the primitive forebrain sub-ventricular zone (SVZ) that persists in adulthood as a mitotic cell layer called subependymal layer (SEL) (Peretto et al., 1999). Once generated the committed, but still undifferentiated, precursors migrate long distances to reach their final destination in the OB. The neural progenitors contained in the adult SVZ have been characterized as true stem cells (Gritti et al., 1999; Gritti et al., 1996; Morshead et al., 1998; Reynolds and Weiss, 1996), since they have:

1. An extensive capacity to self-renew.
2. The ability to undergo asymmetric cell division generating a multilineage progeny (multipotentiality).
3. Capacity to exist in a mitotically quiescent state.

In the hippocampus of several mammalian species (Gage et al., 1995; Gage et al., 1998), cell proliferation leading to neurogenesis has been described in the dentate gyrus granular layer. In the hippocampal subgranular zone, clusters of BrdU-labelled undifferentiated cells are often found in close proximity to dividing endothelial cells located at the tips of the capillaries suggesting that these cells are part of a specific "niche" associated with blood vessels (Palmer et al., 2000). Moreover (van Praag et al., 2002) these newly generated cells in the adult mouse hippocampus have the neuronal morphology, passive membrane proprieties, action potentials and synaptic inputs typical of mature dentate granule cells. These results demonstrate that in the adult mammalian dentate gyrus new functional neurons are continuously integrated into the hippocampal circuitry.

Adult Subventricular Zone Stem Cells

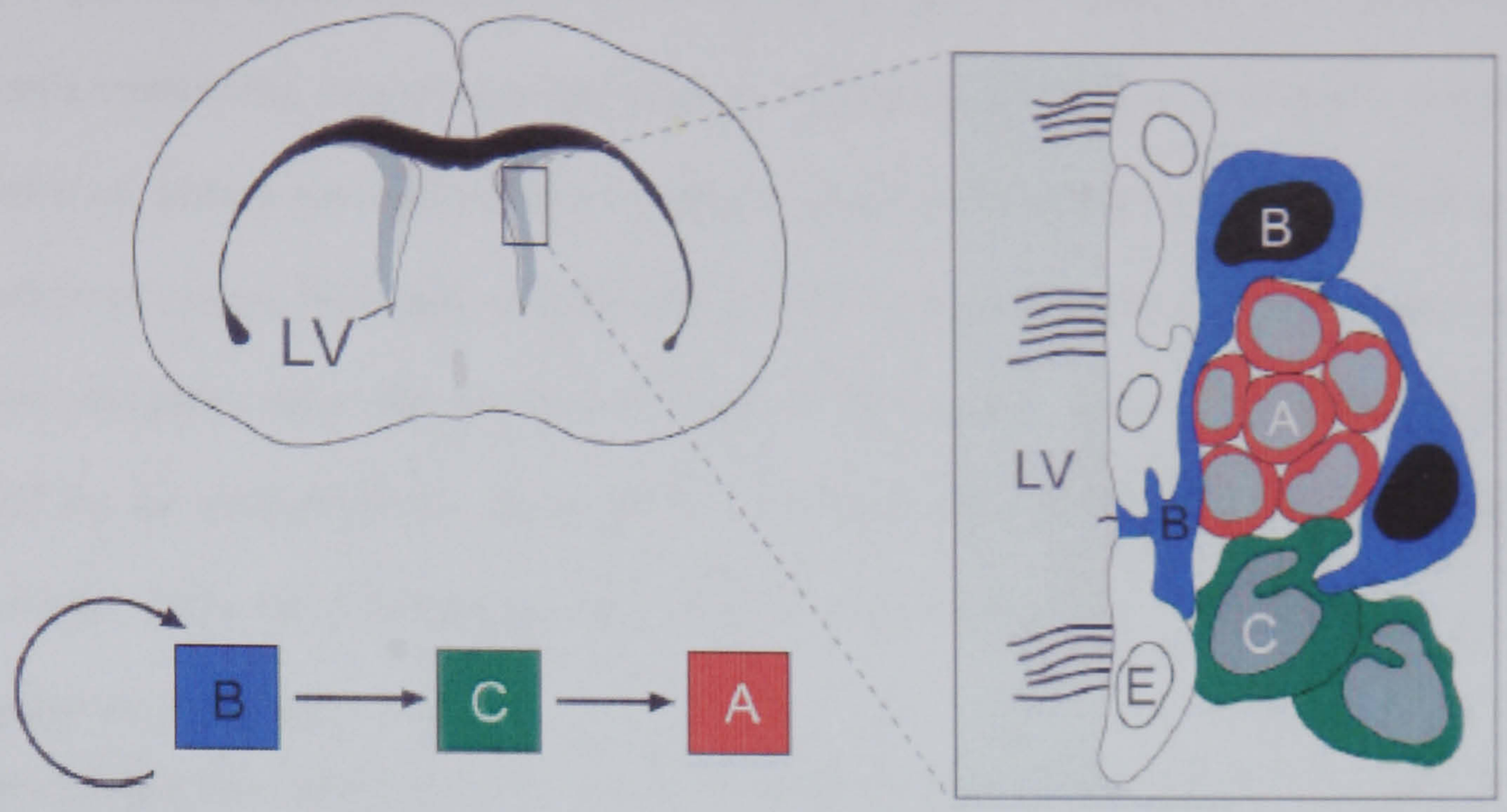
The SVZ is a large germinal zone adjacent to the ependymal layer of the entire lateral telencephalic ventricle. In postnatal (Luskin, 1993) and adult rodents (Lois and Alvarez-Buylla, 1994), cells born in the SVZ migrate along the so-called rostral migratory stream (RMS) to the OB where they differentiate into interneurons. Interestingly, in primates (Gould et al., 1999) the SVZ has been found to generate new neurons for the prefrontal, inferior temporal and posterior parietal cortex, in agreement with the idea that neurogenesis preferentially occurs in brain regions actively implicated in learning and memory. The prefrontal cortex is an association area involved in behavioural plasticity in primates, whereas the olfactory bulbs are regions that have a role in learning and memory in rodents. Self-renewing cells from the SVZ can be isolated and propagated in vitro, and can differentiate into neurons, astrocytes, and oligodendrocytes (Gage et al., 1995; McKay, 1997; Weiss et al., 1996). They are also defined as the self-renewing cell type responsible for maintaining a constant production of OB neurons in vivo. While it is usually thought that stem cells are undifferentiated or primitive, and lack expression of typical markers of more mature cells, it is becoming increasingly clear that stem cells can bear what we

thought to be the biochemical hallmarks of more differentiated cells. In this regard, haematopoietic stem cells, perhaps the most studied stem cells, express what was considered to be lineage-restricted molecules (Hu et al., 1997).

SVZ Cellular composition

As mentioned above, in the adult mouse, cells proliferate along the entire length of the SVZ and then migrate anteriorly to the OB. The migratory neuroblasts (type A cells) travel in chains (Wichterle et al., 1997). These are ensheathed by processes of slowly dividing SVZ astrocytes (type B cells), and scattered along the type A cells are clusters of rapidly dividing progenitor cells (type C cells) (Fig 6).

Fig. 6



Cross section of the adult mouse brain (upper left) and schematic illustration of subventricular zone (SVZ) composition and architecture (expanded region on the right). The chains of neuroblast (A cells) destined for the olfactory bulb migrate through glial tunnels formed by the process of slowly dividing astrocytes (B cells). Focal clusters of rapidly dividing precursors (C cells) are found scattered along the network of chains). SVZ astrocytes (B cells) act as neural stem cells in this region and divide to give rise to rapidly dividing precursors (C cells) that in turn generate the neuroblasts that migrate to the olfactory bulb.

The type A cells are immunopositive for the neuron-specific-β-tubulin antibody (Tuj1), and express a polysialated form of neuronal cell adhesion molecules (PSA-NCAM). Type B cells contain the intermediate filament glial fibrillary acidic protein (GFAP), a typical marker of mature astrocytes. Type C cells are ultrastructurally immature, do not express markers of mature brain cells, and have high mitotic activity. For these reasons type C cells have been proposed as the precursor of type A cells (Doetsch et al., 1997). Adjacent to the SVZ lie the multiciliated ependymal cells, which appear highly differentiated bearing multiple cilia in the cerebrospinal fluid of the ventricular system.

Studies on SVZ stem cells

Two groups have studied the origin of the stem cells generating the granule cells of the olfactory bulb. One has suggested that these stem cells correspond to the differentiated ependymal cells (Johansson et al., 1999); another (Doetsch et al., 1999a) has instead concluded that they are equivalent of the astrocyte-like type B cells expressing GFAP. An accepted notion is that adult stem cells divide very slowly and, for instance, haematopoietic stem cells enter the cell cycle every 1-3 months to maintain haematopoiesis (Cheshier et al., 1999). Regarding the SVZ compartment, the stem cells are the most slowly dividing cells of this region (Doetsch et al., 1999a), and for this reason, an efficient labelling of stem cells requires repeated administration of BrdU or [^{3}H]thymidine. This procedure identifies the stem cells as cells retaining the label for prolonged periods (label-retaining cells, LRCs), whereas mitotically active progenitors dilute it and/or migrate from the region. Because in this kind of experiment some labelled nuclei appear to be very close to or within the ependymal layer, Johansson et al. concluded that ependymal cells are the LRCs, while electron microscopy studies, performed by Doetsch et al., have demonstrated that type B cells close to the ventricular lumen have their nuclei separated from the lumen by a thin process of an adjacent ependymal cell, and such nuclei could be easily mistaken as belonging to the ependymal layer. Moreover, double immunohistochemistry studies

have demonstrated that none of the BrdU labelled nuclei coexpress markers of ependymal cells (CD24) (Doetsch et al., 1999a).

Thus, at present data indicate that the majority of SVZ LRCs are type B cells, and infusion of antimitotic agents (Doetsch et al., 1999b) has established the relationship between type A, B and C cells, in which the developmental lineage is as follows: B to C to A.

In conclusion, type B cells have been confirmed by electron microscopy to be the in vivo LRC, they produce OB neurons, they form multipotent neurospheres in vitro, and their presence is sufficient to regenerate the SVZ after the elimination of rapidly dividing cells. Therefore, the expression of GFAP is not synonymous of glial lineage: in the SVZ, 10% of the GFAP-positive type B cells can form multipotent neurospheres indicating that only a subset of type B cells can serve as stem cells, and obviously specific markers of these cells would be critical for their identification and isolation.

Type B cells, radial glia, and stem cells

Radial glia cells are among the first cells to develop in the ventricular zone (VZ), and their processes extending from the ventricular lumen to the pial surface serve as guides for the neuroblasts radial migration to their final destination in the cortical plate (Rakic, 1972). Once the neurogenesis is over, there is good evidence that radial glia differentiate into brain astrocytes. While most radial glia in the mammalian brain disappear after birth, in many non-mammalian species they persist into adulthood, such as in the avian brain, where they continue to divide throughout life. Recently (Malatesta et al., 2000) it has been reported that radial glia isolated from mouse and rat neocortex can produce both neurons and glial cells *in vitro*, and that radial glial cells correspond to the primary precursors for neurons and glia in the VZ (Noctor et al., 2001). Therefore the embryonic glial cells, which apparently seem the most "differentiated", turn out to be the primary precursor cells. The same findings are true for the SVZ astrocytes of the postnatal brain that have been recognized as the real neural stem cells (type-B cells). The origin of SVZ astrocytes is uncertain, but it seems likely that they originate from the radial glia. In fact in a recent

study (Gaiano et al., 2000) it has been proposed a stem cell lineage: neuroepithelial cells ⇒ radial glia ⇒ astrocytes. In ageement with this hypothesis radial glia cells and neuroepithelial stem cells of the developing neural tube both contact the ventricular and the pial surface and extend a single short cilium into the cerebrospinal fluid. Moreover mammalian radial glia express nestin, an intermediate filament characteristic of neuroepithelial cells and cultured neural stem cells (McKay, 1999).

In conclusion, SVZ type B cells might derive from radial glia cells that retain some neuroepithelial stem cells properties into adulthood, and the SVZ microenvironment might provide signals programming type B cells to continuously generate OB neurons.

Stem cells and *in vitro* culture

Stem cells from the adult SVZ

The stem-like properties of cells isolated from the SEL can be observed by culturing them in serum-free medium with the addition of mitogens such as epidermal growth factor (EGF) or basic fibroblast growth factor-2 (FGF-2). These growth factors induce these cells to expand their proliferative potential and form neurospheres, containing the neural stem cells (Weiss et al., 1996). The expanded stem cell population gives rise mainly to neurons (Peretto et al., 1999) but, under appropriate *in vitro* conditions, generates a variety of progenitor cells able to differentiate into the major cell types of the CNS: neurons, astrocytes, and oligodendrocytes (Weiss et al., 1996).

Until recently, it was thought that there were two classes of SEL-derived neural stem cells, the EGF- responsive and the FGF-2 responsive cells, but recently (Gritti et al., 1999) it has been demonstrated that indeed they are the same cell type that has the capacity to respond to both EGF and FGF-2.

Stem cells from the Rostral Extension and Olfactory Bulb

In vivo SVZ precursors generate primarily committed neuronal precursors that migrate tangentially along the rostral extension (RE) of the SVZ to the olfactory bulb (OB) and constitute the so-called rostral migratory stream (RMS). It has been demonstrated that stem cells can be cultured from the different regions of the RE, including the OB (Gritti et al., 2002). These stem cells show growth profiles and differentiation potentials that are similar, but not identical, to their periventricular cognates. The presence of multipotent stem cells throughout the RE reinforces the idea that the SVZ-RE is a neurogenic compartment displaying a characteristic anatomical and histological organization (Doetsch and Alvarez-Buylla, 1996; Peretto et al., 1999). However, the finding that stem cells have different features at different levels of the adult SVZ-RE reveals a functional heterogeneity of this system.

Stem cells from the adult hippocampus

Multipotent cells similar to the SEL stem cells can be isolated from adult rat hippocampus (Palmer et al., 1997), but these cells are sensitive only to FGF-2; they do not form neurospheres, and they do not undergo long-distance migration in vivo. Moreover, the self-renewal properties of these cells have not been proved by serial sub-cloning analysis. In a recent study (Seaberg and van der Kooy, 2002) it has been proved that while the adult SEL contains true stem cells, the hippocampal SGL cells are committed progenitors. In particular, proliferative cells residing in the hippocampal SGL consist of two types of progenitors cell, one neurogenic and another exclusively gliogenic, and neither of these progenitors is capable of extensive cell renewal. On the contrary, individual proliferative cells of the subependymal layer surrounding the hippocampus shows long-term self-renewal, and they are capable of generating neurons, astrocytes and oligodendrocytes.

Conclusions

Because of the biological potential of stem cells, it is becoming clear how important it would be to dissect the molecular program that underlies NSC proliferation and differentiation. In order to study the transition during differentiation from one cell type to another, investigators need to be able to distinguish between the cell types in a lineage. The mechanisms underling cell fate specification in neural stem cells are of extreme interest, as these mechanisms might hold the key to neural repair strategies. In the ideal setting, one would need to have markers for neural differentiated cells and also for CNS stem cells and lineage committed progenitors, markers that for the moment are lacking (Kornblum and Geschwind, 2001). A cell type can be defined by the complete profile of the expressed genes at that specific stage, and understanding which genes are "on" in stem cells, and which instead start to be expressed as soon as the cells begin to differentiate, will be of great importance in understanding stem cell biology and the therapeutic potential of these cells. This is particularly true now, since papers have reported the use of neural stem cells for the treatment of neurological diseases like Parkinson's disease (Kim et al., 2002) and multiple sclerosis (Pluchino et al., 2003). In order to unravel such molecular programs, my approach has been to use microarray technology to identify novel markers and to study these genes for their sequence and expression patterns in vivo.

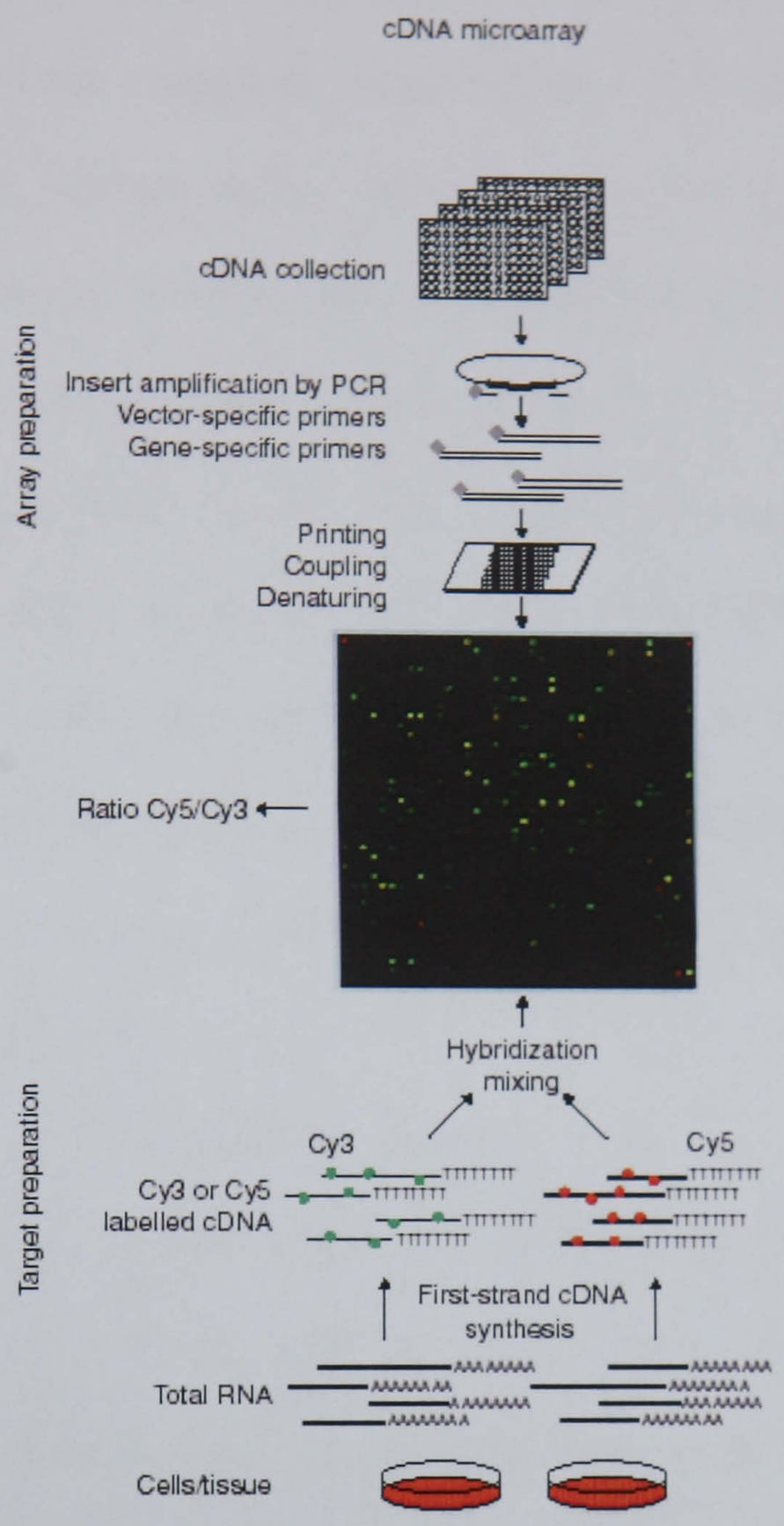
In order to pursue this goal I took advantage of the *Tess* (Telencephalic embryonic subtracted sequences) library (see cDNA Microarray) (Porteus et al., 1992). The hypothesis driving this choice was that this cDNA library is a restricted collection of genes preferentially expressed in the E14.5 telencephalon, and at this developmental stage proliferation, migration and differentiation of neural cells it is actively taking place. Thus, it is reasonable that the same genes will be involved in adulthood, in the biology of adult neural stem cells "niches". Moreover, since a major challenge in microarray experiments is to decide which genes are important for defining a cell type, the use of this dedicated array

based on a specific collection of genes allowed me to avoid the "noise" of genes not related to neurogenesis.

cDNA microarray

DNA arrays are among the most powerful and versatile tools for genomics and genetic research. They allow a systematic analysis of the growing body of sequence information and quantitative measurements of gene expression (messenger RNA abundance) for thousands of genes at a time. The arrays of DNA sequences, immobilized on a solid support (usually glass), are hybridized with cDNA, or RNA. The arrayed material has generally been termed "probe" since it is equivalent to the probe used in a northern blot analysis. The use of different fluorescent dyes (such as Cy3 and Cy5) allows the differential labelling of mRNA from two different cell populations, or tissues, with different colours, which are then mixed and hybridized to the same array, exerting a competitive binding to the arrayed sequences (Fig 7). After hybridization and washing, the slide is then scanned at two different wavelengths, corresponding to the dyes used, and the relative intensity of the same spot (same target sequence) in both channels is compared. The result is a measurement of the ratio of transcript level for each gene represented on the array.

Fig. 7



cDNA microarray overview. Array preparation: inserts from cDNA collection or libraries are amplified. PCR products are printed at specific sites on glass slide. Target preparation: RNA from different tissues or cell populations is used to synthesize single-stranded cDNA. Both samples are mixed and hybridized to the array surface. High-resolution confocal fluorescence scanning of the array with two different wavelengths provides relative signal intensities and ratios of mRNA abundance.

Microarray data typically consist of measurements of spot intensities and intensity ratios. The challenge is then to sieve through this mound of data and find meaningful results for the experimental setting. Replication has been shown to greatly reduce the number of potential false positive results, especially if associated with the reciprocal swapped colours labelling (because of the physical diversity of the dyes used).

Microarrays can be successfully used to address different questions depending on the experimental approach. (i) For example, an investigator can be interested in finding single changes in gene expression that might be key for unravelling specific molecular alterations in a given phenotype. (ii) On the other end, the aim can be to assess the complete gene expression profiles in many samples in order to understand the complex architecture of genetic regulatory networks.

The intrinsic problem of microarray experiments is that they generate long lists of differentially expressed genes, but they provide few clues as to which of these changes are directly involved in a given cellular and tissue phenotype. A generic stimulus/mutation/environmental change can in fact lead to substantial changes in the mRNA level of hundreds of genes, particularly in mammalian systems. A strategy to overcome this limitation is to look for differentially expressed genes that conform to the existing knowledge about how the experimental system might work, but, on the other end, benefits of an unbiased approach will be lost if exploration is limited to our current framework of understanding.

DNA arrays and neurobiology

Recent studies on yeast have shown that expression profiling can provide access to the entire regulatory network in any given physiological process by revealing concerted and genome-wide changes in transcription (Cho et al., 1998; Chu et al., 1998). Furthermore, by inferring that the relative abundance of transcripts is a response to the specific cellular needs, we can predict the function of many previously uncharacterised genes, whose

expression level tightly correlates with the time course of a specific event and transcriptional changes of known genes. Thus, the global transcription profile can be considered a direct representation of a specific cellular phenotype or state.

These characteristics make biological discovery with microarray expression profiling extraordinary powerful for molecular neuroscientists, because it allows the detection of the molecular signature of complex cellular events, such as the cellular commitment to the different neuronal cell fates, the formation and maintenance of appropriate neuronal connections, the molecular identity of neuronal circuits involved in cognitive and behavioural processes, and the characteristics and progression of different neurological diseases.

Examples of microarray analysis in neurobiology

The vertebrate brain is subdivided into anatomically and functionally distinct regions and structures. The dissection of the molecular network underling the development and function of these distinct units represents a critical step for further functional analysis, and will lead to the identification of brain-specific therapeutic targets. Two different studies have analysed the gene expression profiling of specific brain regions using the Affymetrix Murine set of oligonucleotide array (Sandberg et al., 2000; Zirlinger et al., 2001). In particular, Sandberg *et al.* looked at the expression of ten thousand genes in six different brain regions of 129SvEv and C57BL/6 mice. In a similar approach, Zirlinger et al. went further and assessed by in situ hybridization analysis that 60% of the identified genes were expressed in a consistent manner with the array analysis, while 20% were not detected, 13% have ubiquitous expression and 7% were inconsistent with the microarray results. These differences can be mostly attributed, at least in some cases, to sub-optimal probe array design rather than the inaccuracy of the GeneChip method. For both studies, an average of only 0.3-0.5% of the genes show a significant expression difference in a given brain region compared to another, and this can be due to two factors: (i) the cellular

heterogeneity in most brain regions may result in the dilution of rare cell-specific transcripts, (ii) many unknown region-specific genes may not be represented in the available databases and Gene Chips.

Developmental processes have also been subjected to microarray analysis. Mody *et al.* (Mody et al., 2001) have analysed the developmental transcriptional programs of the mouse hippocampus from embryonic day 16 to postnatal day 30 using the Affymetrix Murine set of eleven thousand oligonucleotides, identifying 1926 differentially expressed genes with at least three fold changes in expression level.

In another study Livesey *et al.* (Livesey et al., 2000) compared the expression profile of dissected retinas from wild type and Crx mutant mice, using a dedicated microarray of 960 adult mouse retina cDNA clones. Crx is a photoreceptor-specific homeobox containing transcription factors that controls terminal differentiation of vertebrate photoreceptors, and by looking at these specific retinal cells, Livesey *et al* successfully identified a core set of photoreceptor genes as candidate targets for Crx.

A different strategy has instead led to the identification of genes preferentially expressed in cultures of CNS progenitors (Geschwind et al., 2001). In this study was first performed a subtraction between cultures of neurospheres, containing 3-4% of totipotent stem cell progenitors, and cultures induced to differentiation for 24 hours, thus likely depleted of progenitors. In a subsequent step, about 6000 subtracted cDNAs were spotted onto glass slides and hybridized with the cDNA obtained from neurospheres and differentiated cultures. Recently, the same group has performed a high throughput screening by *in situ* hybridization of the previously identified genes in order to assess their enriched expression directly in the germinal neuroepithelium, and thus validate those expressed in neural progenitors from those expressed in more differentiated cells *in vivo*.

The studies described above adopted different strategies and reached different outcomes. Mody *et al.* (Mody et al., 2001), by undertaking a genome wide screen for developmental

genes, have identified a large panel of potentially interesting transcripts, but the analysis was restricted to the microarray data. In contrast, Livesey *et al.* (Livesey et al., 2000), and Geschwind *et al.* (Geschwind et al., 2001) have instead focused their search for differentially expressed transcripts, by directly comparing between mutant and wild type tissue, or by using a collection of specific transcripts.

Tess chip: "telencephalic-expressed sequence" cDNA array

All the considerations discussed above lead to the decision to focus my analysis on a collection of genes specifically involved in CNS development. In order to achieve this goal, I took advantage of a specific cDNA library realized by subtracting adult telencephalic cDNA from embryonic telencephalic cDNA (Porteus et al., 1992). This subtraction has been performed between E14.5 and adult telencephalon cDNA libraries in order to isolate genes directly involved in the various neurodevelopmental processes that take place in the E14.5 mouse telencephalon, including: (i) genes expressed in the mitotically active undifferentiated cells of the ventricular zone, (ii) genes that are transiently expressed in differentiated cells, (iii) genes that are expressed in transient embryonic structures such as the subventricular zone, subplate and primitive plexiform layer, and (iv) genes that have region specific patterns of expression (Porteus et al., 1992). This library consists of about one thousand different telencephalic-subtracted transcripts (*Tess* sequences) that are exclusively or preferentially expressed in the E14.5 embryonic telencephalon. For this reason I decided to develop a dedicated array by spotting these clones onto glass slides to investigate telencephalic development by microarray analysis. This chip of developmental cDNAs has been used to perform two different types of studies:

- Analysis of the telencephalic gene expression profiles of mice mutant for transcription factors that are involved in forebrain development (Dlx1/Dlx2, Nkx2.1, Pax6, and Ngn1/Ngn2).

- Analysis of the gene expression profile of *in vitro* cultured neural stem cells, progenitor cells and differentiated cells.

The rationale of this array approach was that the use of a collection of developmentally specific and largely uncharacterised cDNAs could be extremely informative for gaining insights into the molecular switches of telencephalic development and neural stem cells biology. The particular strength of this approach is the specific characterization of a subset of the transcriptome corresponding to genes specifically expressed in the brain regions of interest and potentially involved in the processes of proliferation, migration and differentiation of neurons.

RESULTS

Tess array

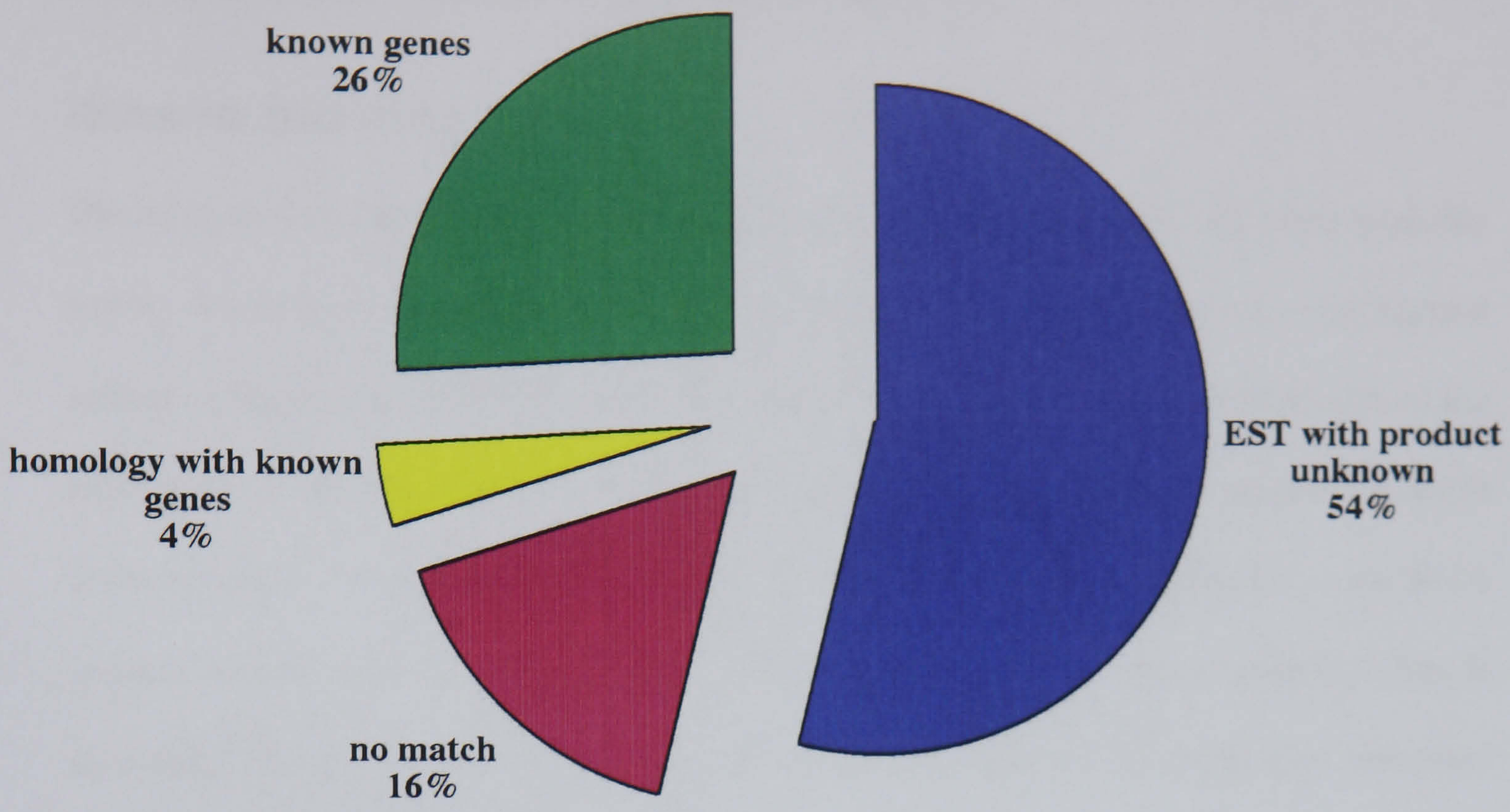
cDNA chip development

The *Tess* (Telencephalic Embryonic Subtractive Sequences, (Porteus et al., 1992)) cDNA microarray was obtained by amplifying, purifying and spotting onto glass slide 1026 different *Tess* cDNA clones. In the process of developing the chip I also decided to include some genes useful as reference for monitoring the gene expression profiling experiments: housekeeping genes (e.g. β-actin, Hprt), telencephalic specific marker genes (e.g. Tbr1, Gad65, Dlx2, Tbr2), and yeast intergenic regions for calibrating the two dyes (see Materials and Methods). The *Tess* cDNAs and the marker genes were spotted in four replicates in each slide, since an appropriate number of replicates in the microarray is of critical importance to perform a reliable statistical analysis of the results.

Sequence analysis of the *Tess* clones.

All the sequences of the *Tess* cDNAs selected to develop the array have been subjected to accurate bio-informatics analysis, and as a result, about 70% of the cDNAs correspond to genes that have not been characterized yet, confirming the relevance of this restricted collection of genes. Fig.8 shows the distribution of the sequences after the bio-informatics analysis.

Fig. 8



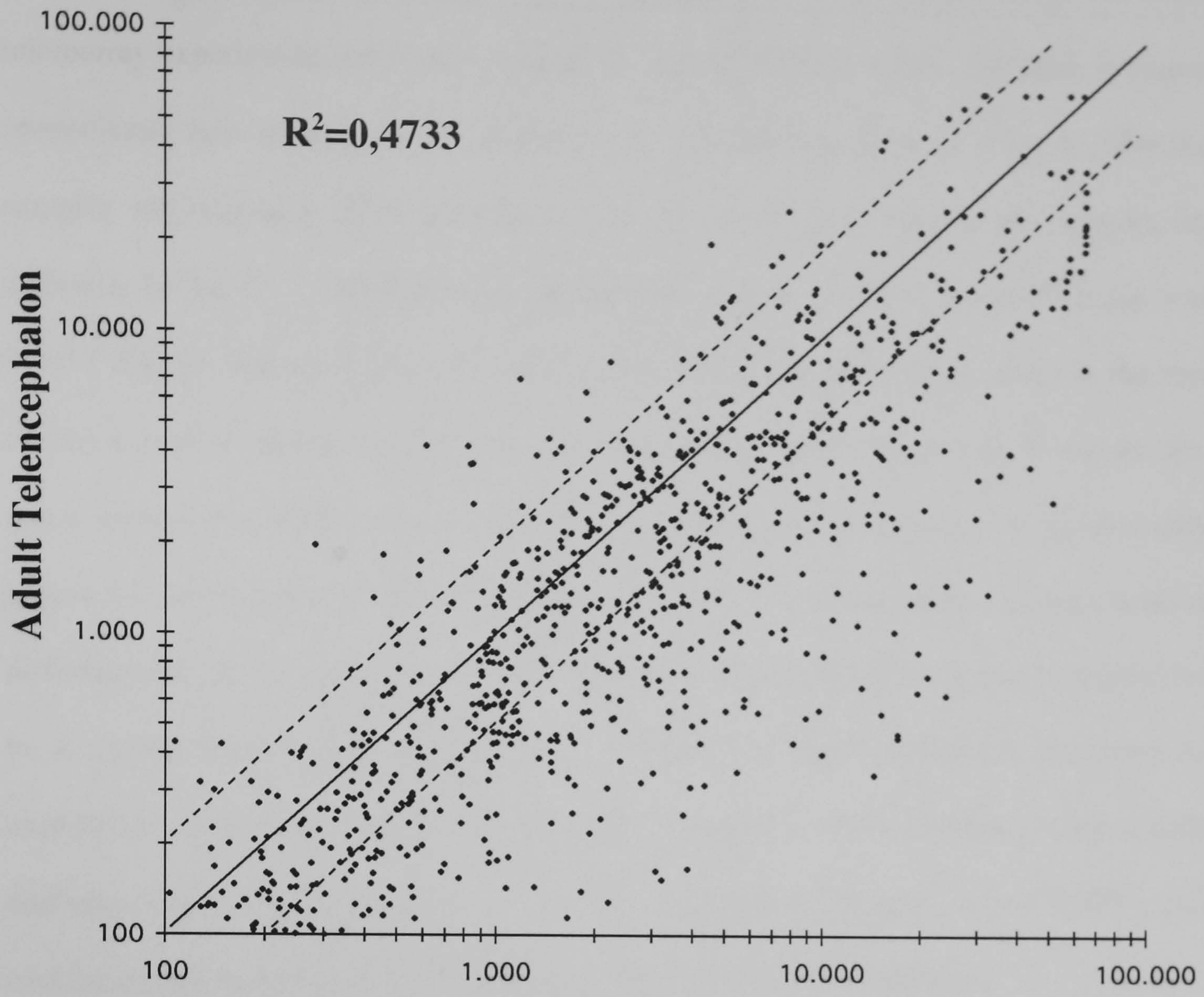
Classification of the one thousand different *Tes* transcripts into categories.

Tess array characterization: E14.5 telencephalic genes

Microarray hybridization

The main goal of this project was the identification and characterization of genes (possibly novel) involved in telencephalic development, and to this end different experimental strategies have been used. One approach was to detect, by microarray hybridization, the relative level of expression of these genes in the E14.5 telencephalon compared to adult telencephalon. Telencephalons from E14.5 and adult mice were dissected, total RNA isolated and the corresponding labelled cDNA hybridized to the *Tess* microarray chip. A dye-swap analysis was also performed, and in Fig.9 is depicted the scatter plot generated from these hybridizations.

Fig. 9

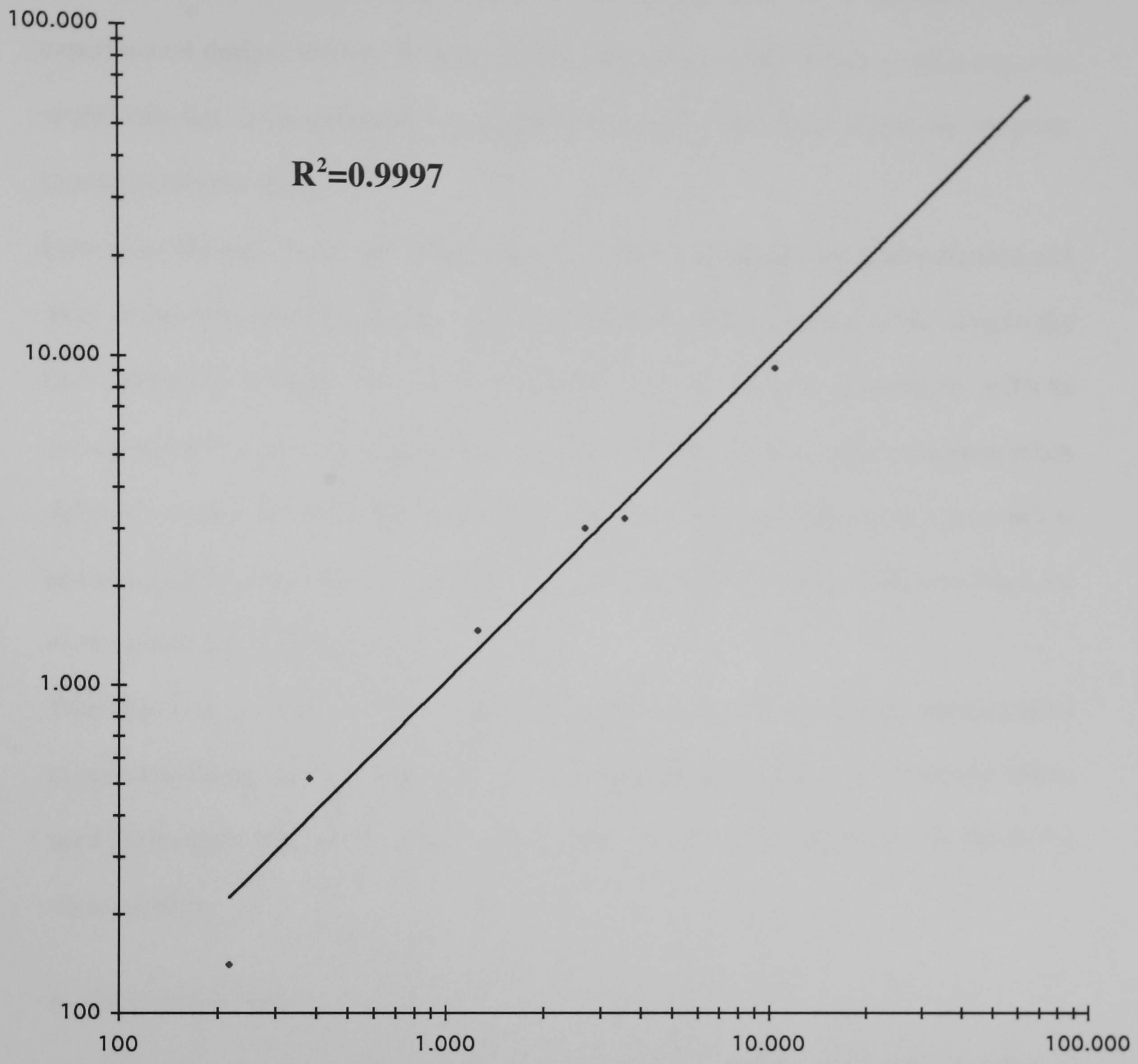


E14.5 Telencephalon

Scatter plot analysis of embryonic (E14.5) telencephalon versus adult telencephalon. Average expression levels (arbitrary units) were calculated from two hybridizations (dye swap analysis). Genes that show at least 2-fold difference are below (E14.5 genes) and above (adult genes) the dotted lines. The genes that are between the two dotted lines are considered equally expressed.

The analysis of the scatter plot chart is an important tool for an accurate assessment of a microarray experiment, and in particular the R^2, or co-efficient of determination, indicates immediately how similar are the samples: a high value (close to 1) indicates that the samples are extremely similar, a low value (close to 0) indicates that the samples are different. In the E14.5 telencephalon/adult telencephalon comparison, the R^2 value was 0,4733 (Fig.9), indicating that the gene expression profile of the *Tess* genes in the two tissues diverge substantially due to the cDNA subtraction between the two tissue. In fact, many array elements/spots lie off the regression line, and correspond to differentially expressed genes between the two tissues. Since the array experiment showed striking differences in gene expression, I decided to carefully assess the procedure of hybridization by analysing the behaviour of the yeast intergenic DNA that I included in the array. As expected for a reliable experiment, the respective values in the two channels were mainly unchanged, and in fact (Fig.10) the R^2 coefficient for this set of genes was of 0,9997, thus confirming the consistency of the procedure of hybridization and analysis.

Fig. 10



Scatter plot analysis of adult telencephalon versus E14.5 telencephalon, considering only the yeast DNA intergenic regions (calibrations) expression levels. Average expression levels of ten calibrations were calculated from two hybridizations (dye swap analysis).

These data demonstrate the specificity of the library and the accurateness of the experimental design: the low R^2 value in the microarray hybridization experiments was specifically due to the differential gene expression in the two tissues, at least for the genes represented in the library.

Data were obtained from two independent E14.5/adult telencephalon hybridizations and were analysed in order to identify array elements/spots whose intensity ratio between the two conditions (embryo and adult) differed from the normal distribution. cDNAs corresponding to selected spots were chosen for further analysis if their expression ratios differed in both hybridizations. Moreover, to be more confident of the data, a Student's t-test was performed on the four replicates for each spots, and a value <0.02 was found for all the genes described here.

Thus this first preliminary experiment confirmed (i) that the microarray hybridization experiments using the *Tess* chip were well designed and performed, and (ii) that the library used to generate this cDNA chip is highly enriched for genes expressed in the E14.5 telencephalon.

In situ hybridizations

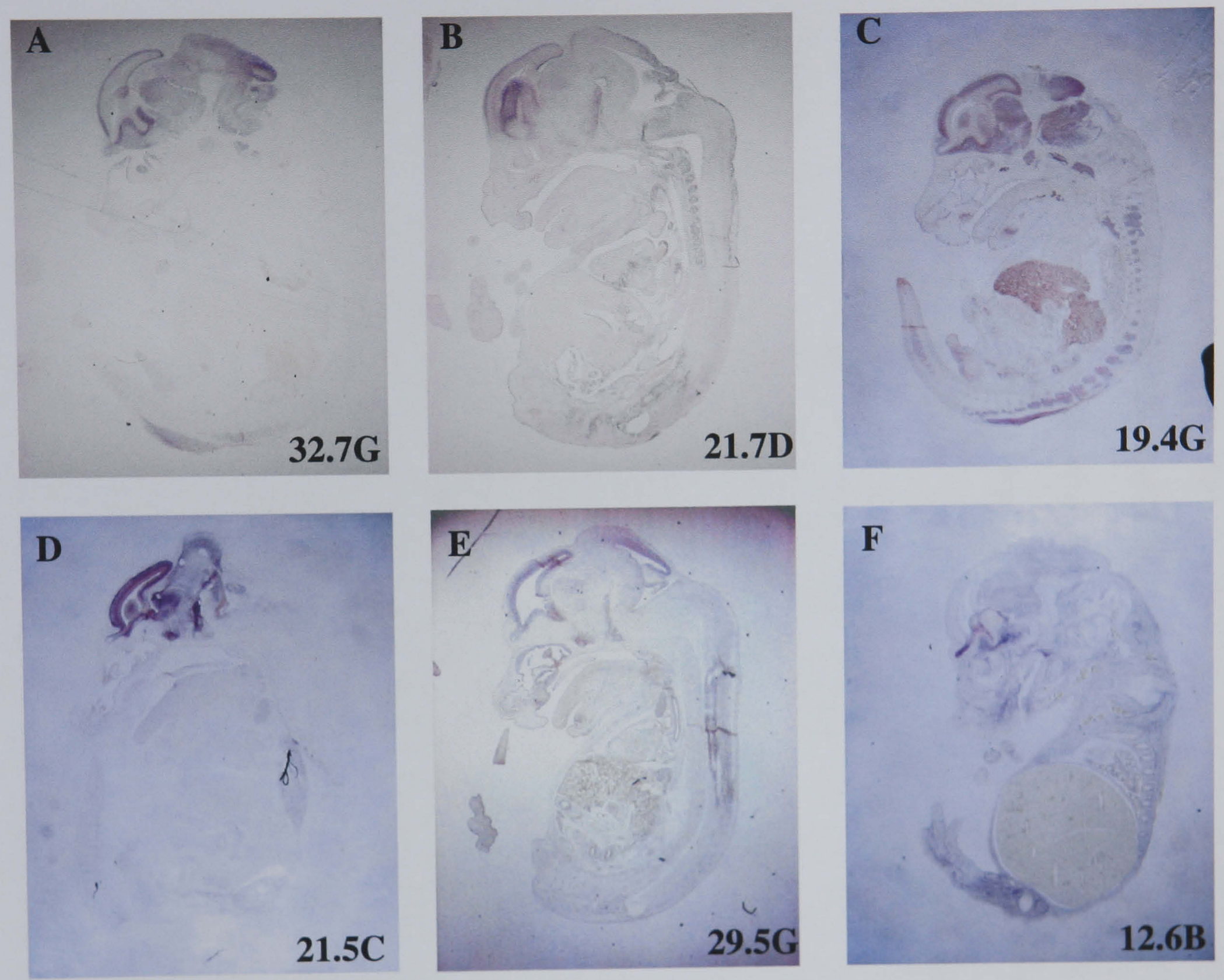
The hybridization experiments using the *Tess* chip served as a screening procedure to select for genes that may play a relevant role in CNS development. Given the highly organized and heterogeneous CNS structure, it is critical to assess where and when a gene is expressed in the developing brain. Thus the method of choice to validate and further investigate the microarray results, especially in neurobiology, is the RNA *in situ* hybridisation. Using this procedure it is possible to verify the expression pattern of the genes found to be differentially expressed by the *Tess* cDNA chip analysis. First, I analysed, by RNA *in situ* hybridisation, genes that were highly up regulated at E14.5 (from 4,40 to 36,15 times: Table 1), and Fig.11 shows the expression patterns of some selected genes.

Table 1:

Genes highly up-regulated at E14.5

Name	Fold	Description	Sequence
32.7G	36,15 ± 1,45	Novel	Mm.275522
21.7D	28,68 ± 3,12	Novel	Chr5B1
19.4G	22,38 ± 2,02	CD24a	NM_009846
21.5C	19,42 ± 1,93	Sox4	NM_009238
29.5G	9,99 ± 2,33	Hmgb2	NM_008252
12.6B	4,40 ± 0,60	Mest/Peg1	NM_008590

Fig. 11



RNA *In situ* hybridizations on WT mouse embryos of the Tes cDNAs up-regulated in the E14.5 telencephalon. The images correspond to sagittal sections of E14.5 mouse embryos hybridized to digoxigenin-labeled RNA probes. See results for description

The genes reported in Fig.11 showed interesting expression patterns. For instance, a first transcript is broadly detected throughout the telencephalon (21.5C, Fig.11D), whereas other genes are specifically expressed in the basal telencephalon (21.7D, Fig.11B), or in the basal telencephalon and olfactory bulb (12.6B, Fig.11F). Clone 29.5G (Fig.11E) is instead expressed in the proliferative zones of both pallium and subpallium. Clone 32.7G has a different expression pattern (Fig.11A), since its signal is localized in the post-mitotic mantle of both pallium and sub-pallium. Clone 19.4G has an analogous distribution (Fig.11C), being expressed in the same differentiated regions, albeit more broadly.

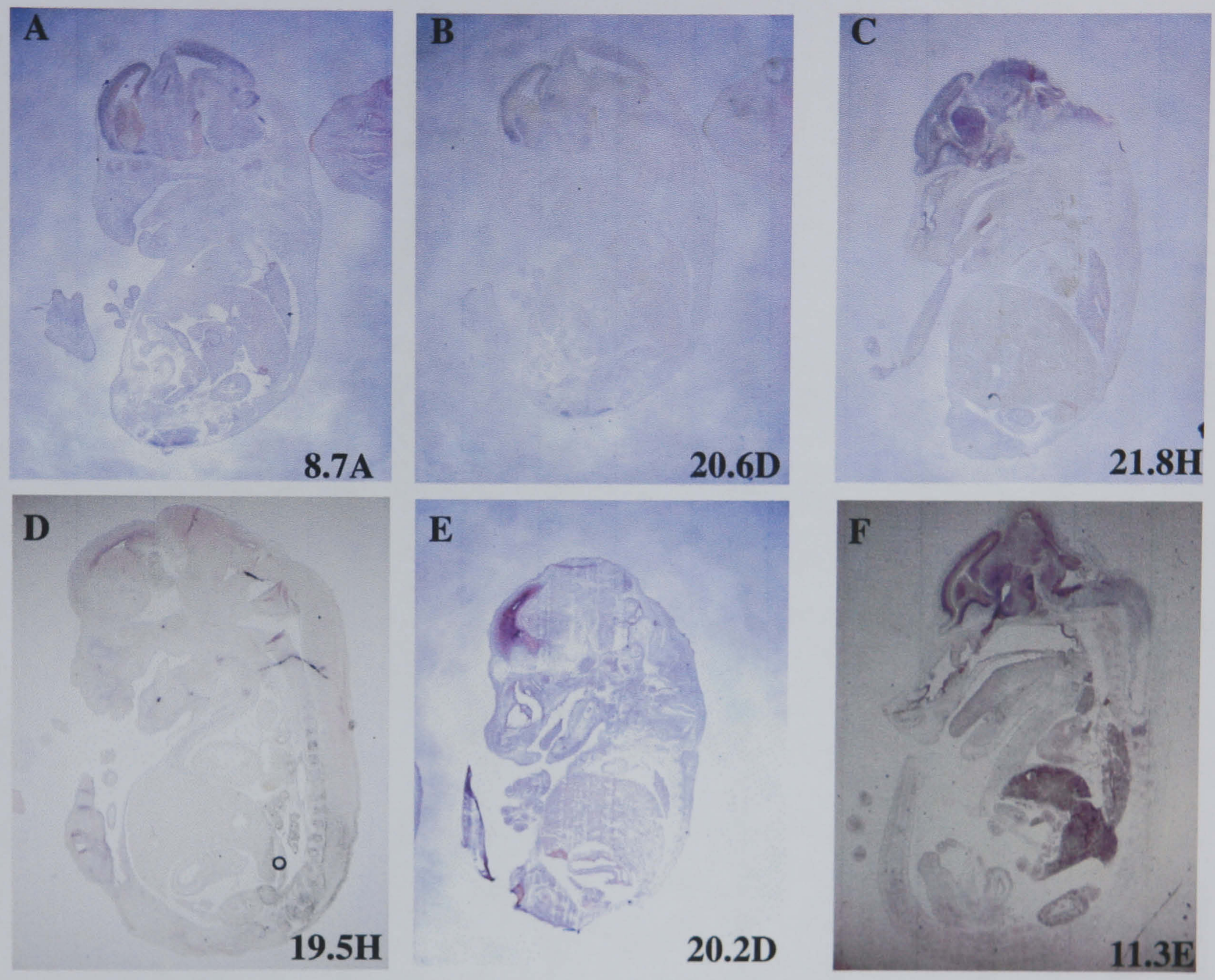
Subsequently the expression patterns of the genes expressed from 2,02 to 3,73 times more at E14.5 (Table 2) were assessed by RNA *in situ* hybridization. Fig.12 shows the expression patterns of some genes.

Table 2:

Genes moderately-regulated at E14.5

Name	Fold	Description	Sequence
8.7A	3,73 ± 0,17	Novel	Mm.22380
20,6D	3,43 ± 0,55	Novel	Mm.132447
21.8H	3,28 ± 0,24	Novel	Mm.142352
19.5H	2,53 ± 0,12	Novel	AA387303
20.2D	2,31 ± 0,40	Cdo	Mm.80509
11.3E	2,02 ± 0,07	Crb1	NM_133239

Fig. 12



RNA *In situ* hybridizations on WT mouse embryos of the Tes cDNAs up-regulated in the E14.5 telencephalon. The images correspond to sagittal sections of E14.5 mouse embryos hybridized to digoxigenin-labeled RNA probes. See results for description.

Although these genes are not as differentially expressed as the previous ones they showed very specific expression patterns. Clone 19.5H (Fig. 12D), for example, is exclusively expressed in the VZ/SVZ of the dorsal telencephalon. The clones 8.7A and 20.6D are characterised by a weak signal (Fig.12A and B), but both have a specific expression in the mantle zone of the dorsal telencephalon, and clone 20.6D shows also a gradient of expression that is high anterior and low posterior. Their relative up-regulation at E14.5 compared to the adult, and their low expression level, detected by *in situ* hybridization, may suggest a minor role in telencephalic development. Nevertheless they are specifically expressed in the CNS (the rest of the embryonic structures were negative) and, at least for 20.6D (Fig.12B), exclusively localised in the dorsal telencephalon.

Analysis of mouse models of telencephalic development

Rationale

The characterization of the developmental molecular networks organised and controlled by transcription factors is a major challenge for functional genomics (Tavazoie et al., 1999; Wen et al., 1998). This kind of analysis has been possible in yeast and in mammalian cell lines *in vitro* (Chu et al., 1998; Holstege et al., 1998; Spellman et al., 1998), but it would be particularly informative if it could be applied also directly to tissue samples, as cell lines typically exhibit changes in both gene expression and genome structure upon cultivation *in vitro*.

In the embryo, different parts of the telencephalon express a variety of regulatory genes that instruct development in a region-specific manner. Many transcription factors are in fact expressed in telencephalic sub-regions, and experiments on relevant null-mutant mice have contributed to unravelling the role of these factors (Schuurmans and Guillemot, 2002).

For this reason, I decided to assess (through cDNA microarray analysis) the expression profile of specific *Tess* genes in a few mice mutant for transcription factor genes. The identification of the genes downstream of these transcription factors should be of great interest for the comprehension of how these DNA binding proteins control telencephalic development. Table 3 shows the list of telencephalic mutant mice used in this study (for a description of their phenotype, see Introduction).

Table 3

Gene	**Gene Description**	**Telencephalic expression**
Dlx1/Dlx2	Homeobox	Subpallium
Ngn1/Ngn2	Basic helix-loop-helix	Pallium
Pax6	Paired-type homeobox	Pallium
Nkx2.1	Homeobox	Subpallium

These mouse models have been carefully chosen in order to study expression profiles of different regions of the telencephalon. For instance, I studied the transcriptional profile of the developing dorsal telencephalon through the analysis of mice mutant for Pax6 and Ngn1/Ngn2, and of ventral telencephalon through the analysis of Nkx2.1 and Dlx1/Dlx2 mutant mice. Furthermore the analysis of sub-pallial gene expression focused on different regions and different populations, since Nkx2.1 expression is restricted to the MGE, in both progenitors and differentiated cells, whereas Dlx1 and Dlx2 are expressed mainly in the SVZ of MGE and LGE. Similarly, in the dorsal telencephalon Pax6 and Ngn1/Ngn2 are expressed in a partial overlapping territory, since Pax6 operates also at the LGE/MGE border in maintaining the LGE identity.

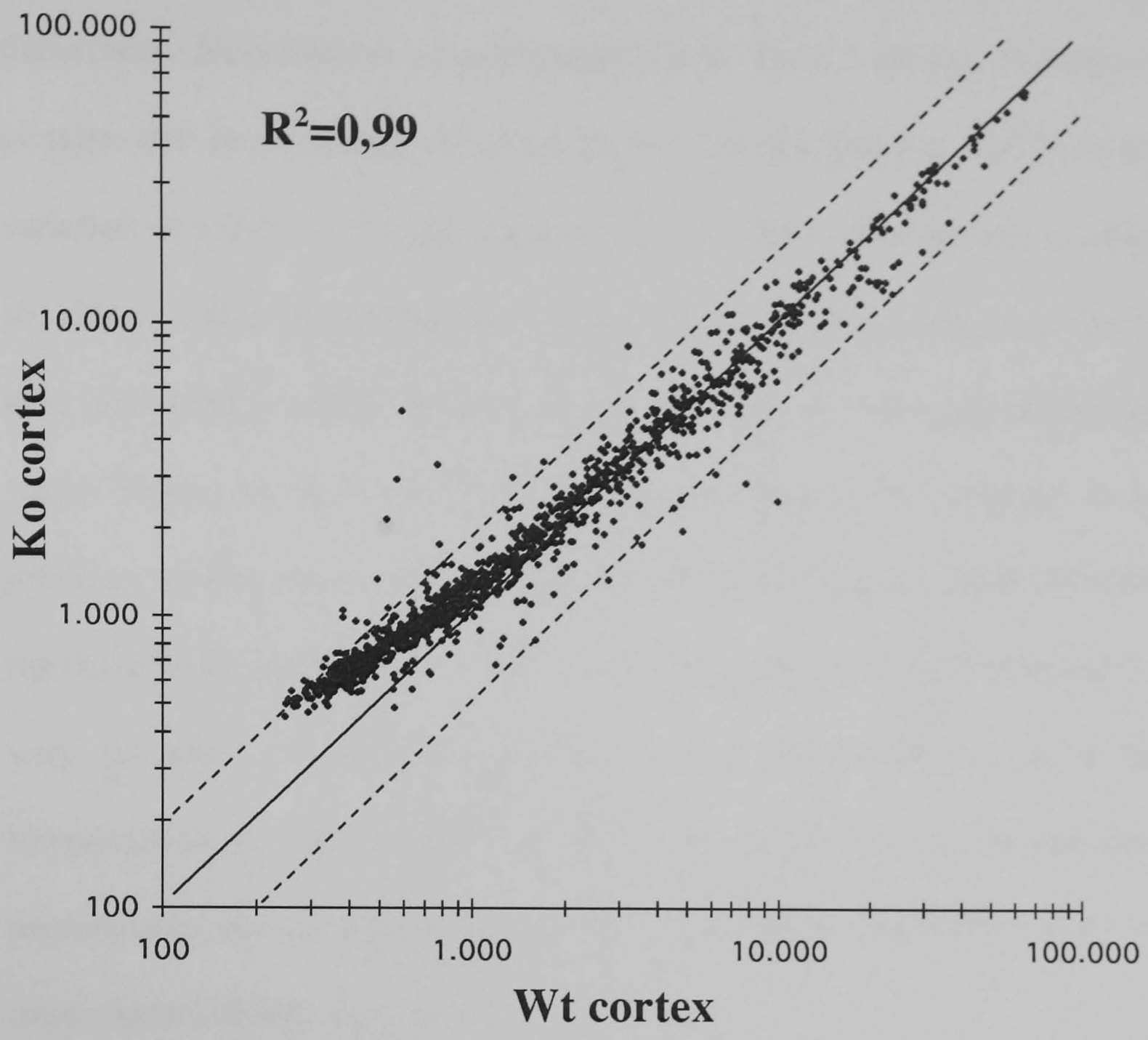
Ngn1/Ngn2 double mutants analysis.

Microarray analysis.

In order to identify components of the transcriptional network controlled by the bHLH transcription factors Ngn1 and Ngn2, I compared the gene expression profile of the Ngn1/Ngn2 mutant embryonic pallium with that of the wild type littermates, using the *Tess*

cDNA microarray. To probe the array, cDNA was synthesized from E14.5 pallium of Ngn1/Ngn2 -/- and WT embryos and labelled by dendrimer technology (see Materials and Methods). Data from two independent wild type versus Ngn1/Ngn2 mutant hybridizations were obtained and analysed to identify the array elements whose detection ratio between the two conditions differed at least two times from the normal distribution. Only the cDNAs that consistently differed from the normal distribution in both hybridizations were analyzed further. Fig.13 shows the scatter plot analysis (on logarithmic axes) of the microarray hybridization, including the linear regression line and the co-efficient of determination (R^2).

Fig. 13



Scatter plot analysis of WT versus Ngn1/2 -/- cortex. Average expression levels (arbitrary units) were calculated from two hybridizations (dye swap analysis). Genes that show at least a 2 fold difference are below (down regulated in KO) and above (up-regulated in KO) the dotted lines. The genes that are between the two dotted lines are considered equally expressed.

In this experiment the co-efficient of determination has a value very close to 1 (0,98), meaning that the gene expression profiles of the two tissues were similar, with only a few differences. Nevertheless, several clones showed a at least two fold increase or decrease consistently in both hybridizations in the mutant pallium, and by t-test analysis the variation was statistically significant ($p<0,02$ in a two tailed analysis). Table 4A and 4B list the cDNAs clones identified, with their relative sequence analysis. In this list are included also some markers originally not present in the library but added as experimental controls, which helped to verify the hybridization procedure. For instance, in agreement with previous studies done by *in situ* hybridization (Fode et al., 2000), Gad65 was found up regulated in the mutant cortex, and Tbr2 down-regulated. The Gad65 and Tbr2 changes are very relevant, not only for the reason that they represent good controls for the hybridization, but also because they are good regional markers for subpallium and pallium respectively, and indicate a likely dorsal to ventral transformation of the telencephalon in these mutant (Fode et al., 2000).

Table 4:

Clones differentially expressed between Ngn1/2 -/- and Wt developing cortex

A. Clones up regulated in mutant cortex

Clone	Fold	Description	Sequence
28.8E	9,02 ± 2,79	Novel	Chr.2
31.5E	5,04 ± 2,37	Novel	Mm.94021
Gad65	4,36 ± 0,66	-	-
Pax6	2,84 ± 0,46	-	-
7.5F	2,72 ± 0,52	Novel	BC022652
31.11A	2,26 ± 0,60	Novel	AK035046
8.9E	2,19 ± 0,84	Novel	AK076994
12.3H	2,13 ± 0,50	Novel	Chr.6B1

B. Clones down regulated in mutant cortex

Clone	Fold	Description	Sequence
18.7E	3,98 ± 0,33	Novel	Chr.16
29.2E	2,70 ± 0,21	Phosducin-like	NM_026176
26.9E	2,42 ± 0,11	Trim 32	NM_053084
13.12G	2,30 ± 0,29	Novel	BQ555930
6.7H	2,03 ± 0,25	Novel	AK046100
Tbr2	1,92 ± 0,30	-	-

As showed in table 4, nine out of eleven genes (markers excluded) correspond to uncharacterized genes (ESTs or simply genomic sequences), confirming once again the usefulness of the *Tess* cDNA subtractive library as a tool for discovering novel genes involved in telencephalic development.

Sequence analysis

The next obvious step was the identification of the genes corresponding to the differentially expressed *Tess* transcripts, since the *Tess* cDNAs contain mainly the 3' untranslated region. The first approach was to perform DNA sequence homology searches (BlastN analysis) in the public databases such as the NCBI (National Center for Biotechnology Information) or the UCSC (University of California Santa Cruz) (see Materials and Methods for details). If the *Tess* cDNA sequence didn't correspond to any

known gene, a second step involved the use of the corresponding cluster of ESTs (usually a UniGene Cluster) to recognize a possible open reading frame (ORF) and search protein public databases (BlastP analysis). Alternatively the sequences of the ESTs cluster digitally translated in all six reading frames were used as a query to interrogate the protein databases. When a putative protein sequence was identified, the SMART program of the EMBL was used in order to characterize its domains. Sometimes the bioinformatics analysis didn't allow the identification of any ESTs corresponding to the *Tess* clone of interest. In this case it was necessary to do an additional search of the mouse, rat, *Drosophila* and human genomic databases in order to identify all possible transcripts (also predicted transcripts) that map around the *Tess* sequence allowing the design of oligonucleotide primers useful for the tentative identification, by RT-PCR, of the corresponding gene.

The sequence analysis of the differentially expressed clones (Table 4) revealed a high percentage of transcripts corresponding to novel genes (9/11). Of these nine genes, only one (7.5F) had a significant homology (75% in the protein sequence) with the human protein *ovarian zinc finger protein* (HOZFP), which contains the Zinc Finger NFX domain characteristic of the transcriptional repressor Nfx1. For three clones it was possible to find only the corresponding genomic sequence: clones 28.8E, 12.3H and 18.7E. Five clones corresponded to single ESTs, or belonged to UniGene EST Clusters, and had no sequence homologies to any known genes. Interestingly three of these clones mapped onto introns of known genes: clone 31.11A in the first intron of Alcam, a gene encoding for a cell adhesion molecule; clone 8.9E into the second intron of Gpi1 gene, a phosphoglucose isomerase; clone 6.7H into the forth intron of Apbb2, the gene encoding for the amyloid beta (A4) precursor protein-binding.

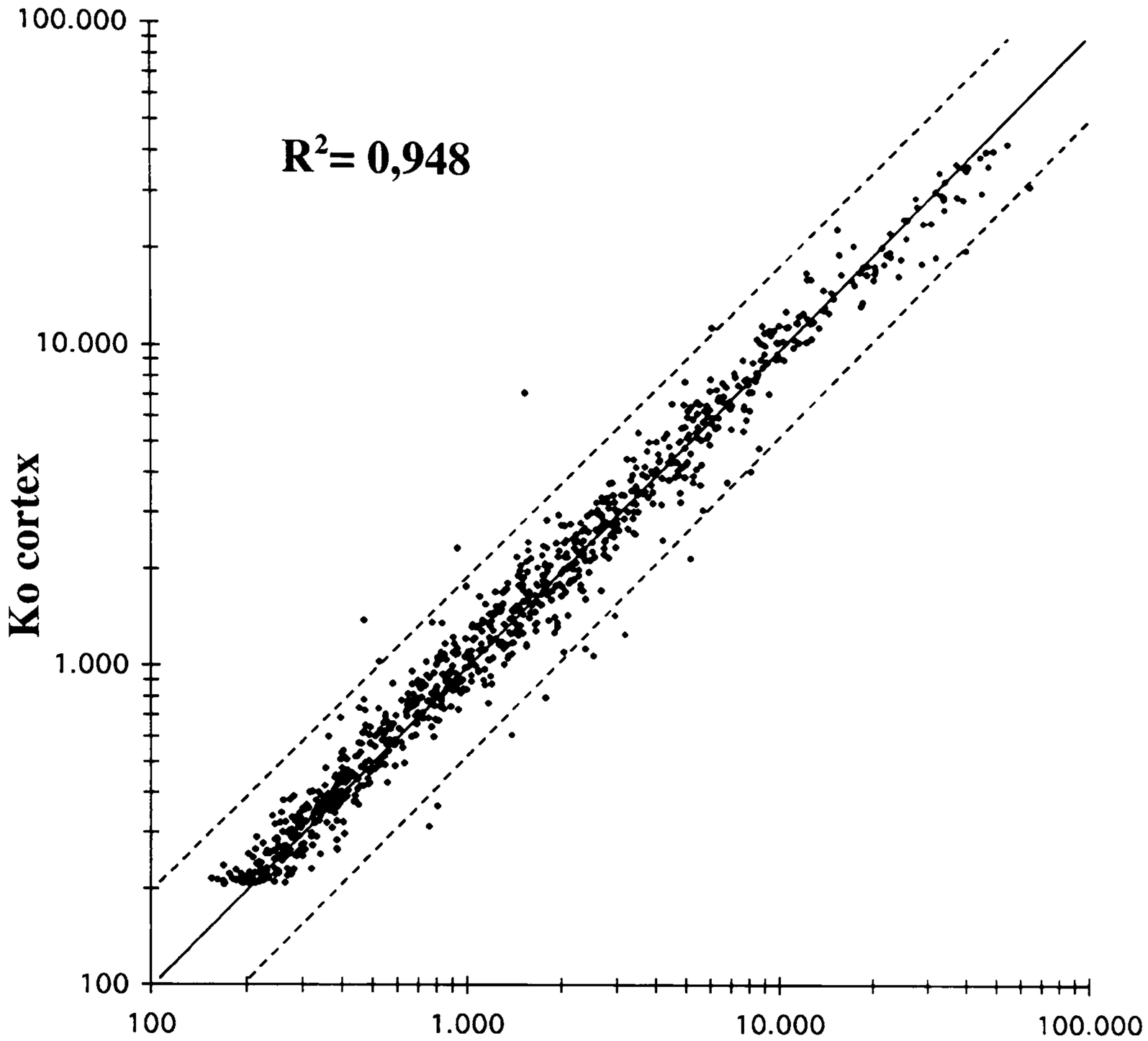
Clone 29.2E corresponds to the known gene Phosducin-like 1, a protein that selectively binds G-protein β/γ subunits, and it is mainly expressed in the neural retina, while clone 26.9E was found to correspond to Trim32, a putative E3-ubiquitin-ligase gene.

Pax6 mutant analysis

Microarray analysis

In order to identify components of the molecular network controlled by the Pax6 transcription factor, I studied the gene expression profile of the mutant animals in comparison with that of wild type littermates using the *Tess* cDNA microarray. To probe the array, cDNA was synthesized from E14.5 pallium of Pax6 -/- (Sey/Sey mouse, a natural mutant with a stop codon in the Pax6 gene) and wt embryos, and labelled as described above for Ngn1/Ngn2 analysis. Data from two independent hybridizations were generated, and analysed in order to identify the array spots whose intensity ratio between the two conditions differed from the normal distribution in both hybridizations by at least two times. Figure 14 shows the scatter plot analysis (on logarithmic axes) of the microarray hybridization data, including linear regression line and the co-efficient of determination (R^2).

Fig.14



Scatter plot analysis of Wt cortex versus Pax6 -/- cortex. Average expression levels (arbitrary units) were calculated from two hybridizations (dye swap analysis). Genes that show at least a 2 fold difference are below (down regulated in KO) and above (up-regulated in KO) the dotted lines. The genes that are between the two dotted lines are considered equally expressed.

The co-efficient of determination was 0.948, meaning that the gene expression profiles of the two tissues were quite similar, showing only a few differences. In fact for only a few clones the expression level in both hybridizations in the mutant pallium demonstrated an increase, or decrease, of at least 2 fold, with a significant t-test analysis ($p<0,02$ in a two tailed analysis). Table 5A and B show the list and sequence analysis of the differentially expressed genes. As for the Ngn1/Ngn2 study, in this analysis some marker genes were present in the array, and among these Gad65 was found up regulated in mutant cortex, whereas Tbr2 and Tbr1 were down-regulated, confirming what was previously shown by Stoykova *et al.* (2000) by *in situ* hybridization.

Table 5:

Identity of clones differentially expressed between Pax6 -/- and WT developing cortex

A. Clones up regulated in mutant cortex

Clone	Fold	Description	Sequence
28.8E	4,34 ± 0,93	Novel	Chr.2
31.5E	3,15 ± 0,82	Novel	Mm.94021
Gad65	2,84 ± 0,63	-	-
8.12C	2,02 ± 0,43	Vrk1	NM_011705
23.10E	1,98 ± 0,25	Novel	Mm.111789
19.3E	1,76 ± 0,46	Foxp2	NM_053242.2

B. Clones down regulated in mutant cortex

Clone	Fold	Description	Sequence
Tbr2	2,52 ± 0,74	-	-
9.10F	2,39 ± 0,64	Novel	Mm.209111
18.9G	2,35 ± 0,91	Top IIα	NM_011623
Tbr1	2,1 ± 0,47	-	-
6.7H	2,07 ± 0,27	Novel	AK046100
13.12G	2,03 ± 0,2	Novel	BQ555930
18.6C	1,92 ± 0,19	Novel	Mm.103539

As shown in Table 5, of the ten *Tess* genes differentially expressed, seven didn't correspond to any known gene, but only to ESTs and genomic sequences.

Sequence analysis

An identical bioinformatic approach used in Ngn1/Ngn2 mutant analysis was used to identify the genes corresponding to the differentially expressed *Tess* clones.

The sequence analysis of the differentially expressed clones (Table 5) revealed that most of the transcripts correspond to novel genes (7 out of 10). For clone 28.8E it was possible to find only the corresponding genomic sequence in the databases. Six clones corresponded to ESTs or belonged to UniGene Clusters, but showed no sequences homologies to any known genes. Clone 8.12C corresponded to a known gene, the vaccinia related kinase 1 (Vrk1). Clone 19.3E was Foxp2 gene, a member of the Fox family of winged-helix/forkhead transcription factors. This gene is most highly expressed in the developing

and mature basal ganglia (Ferland et al., 2003) and is responsible, if mutated, for a form of dyslexia. Clone 18.9G corresponded to Topoisomerase II α (Top IIα).

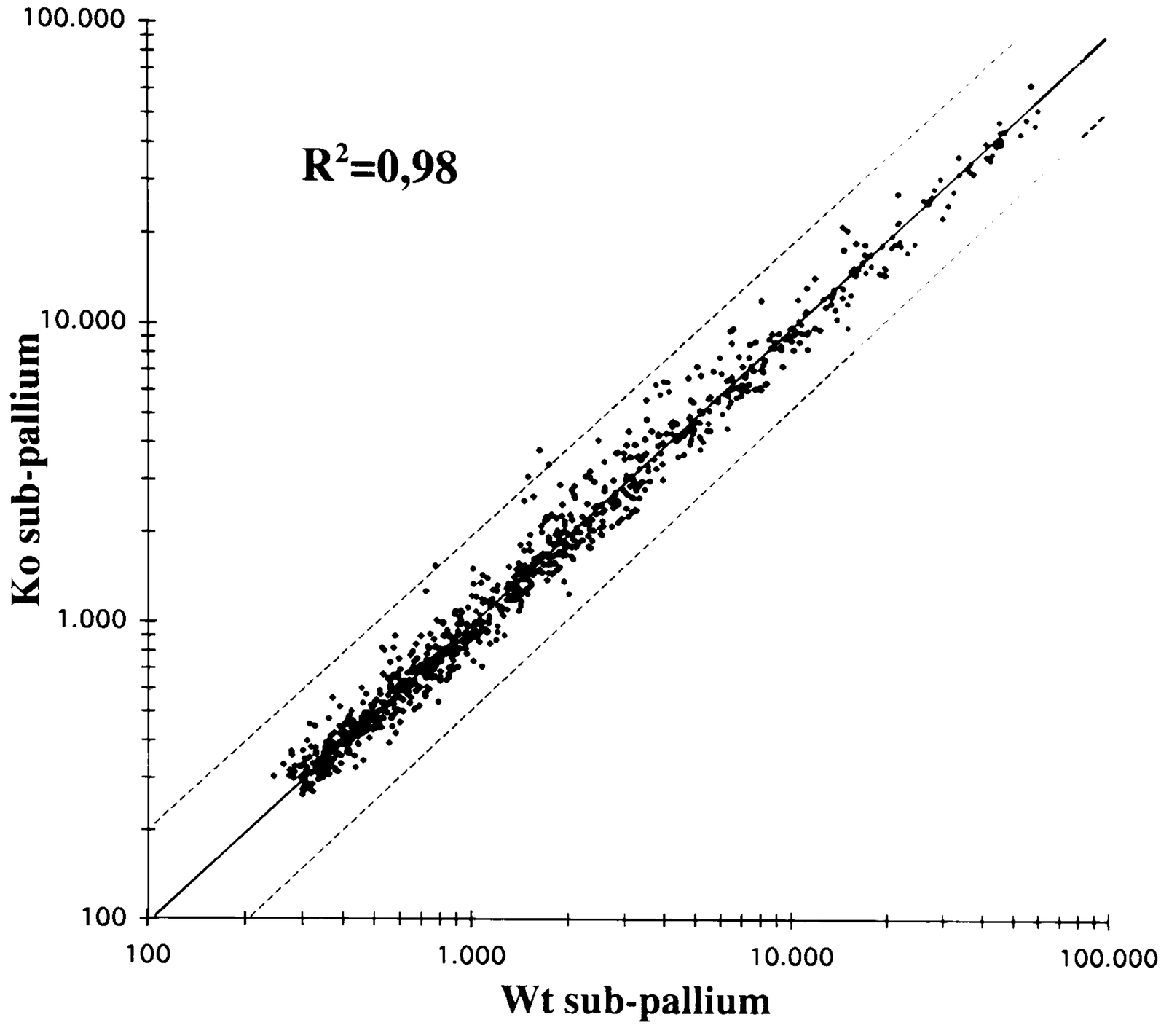
Notably clones 28.8E and 31.5E were also previously found to be up regulated in the mutant pallium of the Ngn1/Ngn2 mutant mice (Table 4).

Nkx2.1 mutant analysis

Microarray analysis

In order to identify the molecules interacting or controlled by the Nkx2.1 transcription factor, the gene expression profile of mutant animals was compared with that of wild type littermates, using the *Tess* cDNA microarray. To probe the *Tess* array, cDNA was synthesized from E14.5 subpallium from Nkx2.1 -/- and WT embryos and labelled as described above. Data from two independent hybridizations were analysed to identify the array spots that differed between the two conditions by at least two times from the normal distribution in both hybridizations. The analysis of this mutant had revealed few and very subtle changes in gene expression. Fig.15 shows the scatter plot analysis (on logarithmic axes) of the microarray hybridization, including a linear regression line and the co-efficient of determination (R^2).

Fig. 15



Scatter plot analysis of Wt sub-pallium versus Nkx2.1 -/- sub-pallium. Average expression levels (arbitrary units) were calculated from two hybridizations (dye swap analysis). Genes that show at least a 2 fold difference are below (down regulated in KO) and above (up-regulated in KO) the dotted lines. The genes that are between the two dotted lines are considered equally expressed.

The co-efficient of determination value was 0.98, meaning that the gene expression profiles of the two tissues were very similar, showing very few differences. In fact only two clones in both hybridizations in the mutant subpallium showed a statistically significant ($p<0,02$ in a two tailed analysis) increase of at least 2 fold (Table 6). One of these genes was Emx2, a gene normally expressed in the dorsal telencephalon.

Table 6:

Clones differentially expressed between Nkx 2.1 -/- and Wt developing sub pallium: clones up regulated in mutant sub pallium

Clone	Fold	Description	Sequence
Emx2	2,32 ± 0,90	-	-
12.6B	2,06 ± 0,45	Mest/Peg1	NM_008590

No clones were found to be down regulated in the mutant sub pallium.

Sequence analysis

The only *Tess* clone found differentially expressed in this analysis was 12.6B. This corresponds to Mest/Peg1, a paternally expressed gene which was also found up-regulated in undifferentiated adult neural stem cells (see neural stem cells results). This gene is normally expressed in the proliferative zone of the embryonic olfactory bulb and the ventral telencephalon.

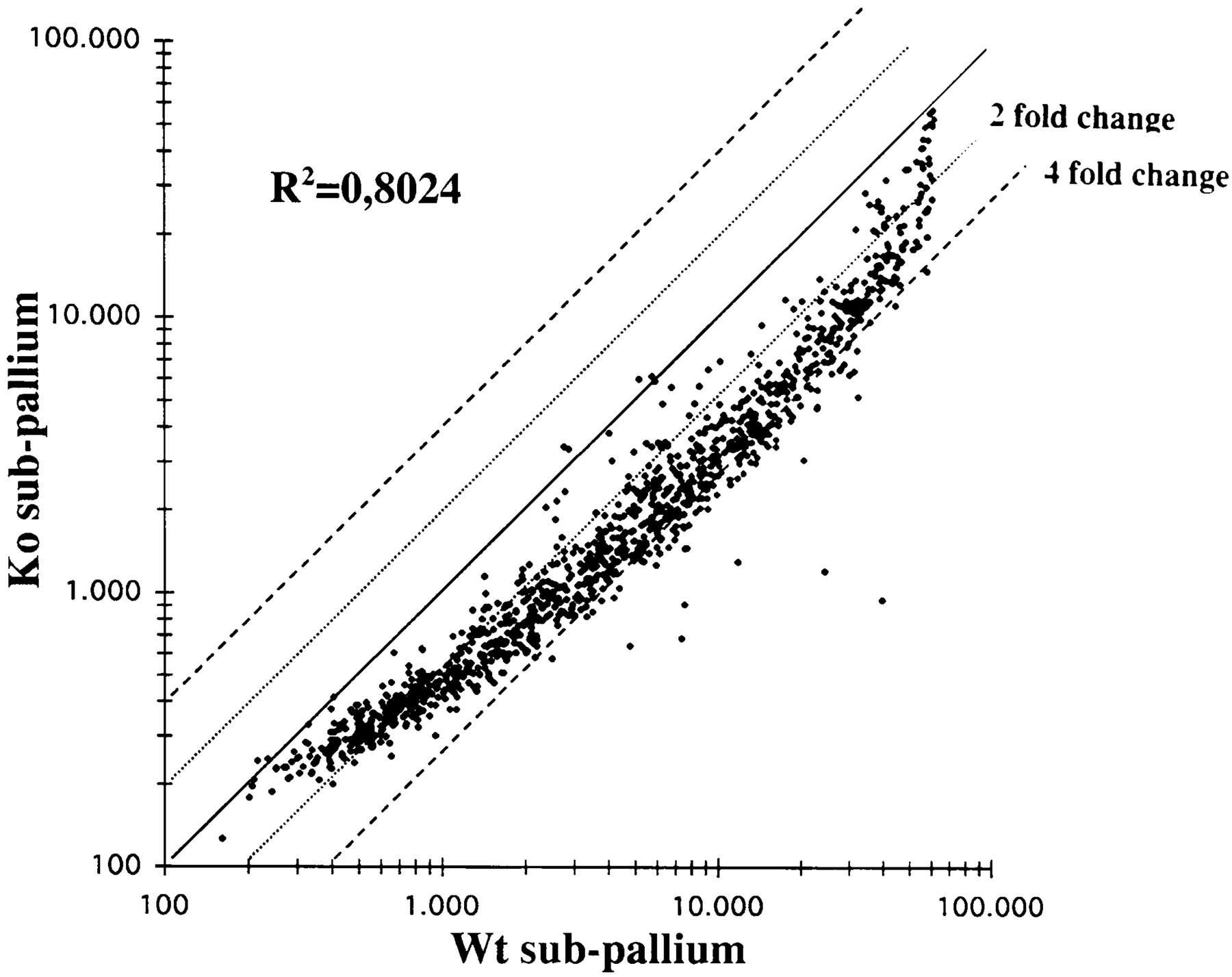
Dlx1/Dlx2 mutant analysis

Microarray analysis

In order to identify the components of the molecular network regulated by the Dlx1 and Dlx2 transcription factors, the gene expression profile of the Dlx1/Dlx2 mutant animals was compared with that of wild type littermates, using the *Tess* cDNA microarray. To probe the array, cDNA was synthesized from E14.5 subpallium of Dlx1/Dlx2 -/- and wt animals and labelled by dendrimer technology (see Materials and Methods). Two

independent wild type versus Dlx1/Dlx2 mutant hybridizations were performed. The spots that differed from the normal distribution in both hybridizations by at least two fold were further analysed. Figure 16 shows the scatter plot analysis (on logarithmic axes) of the microarray hybridization experiments, including a linear regression line and the co-efficient of determination (R^2).

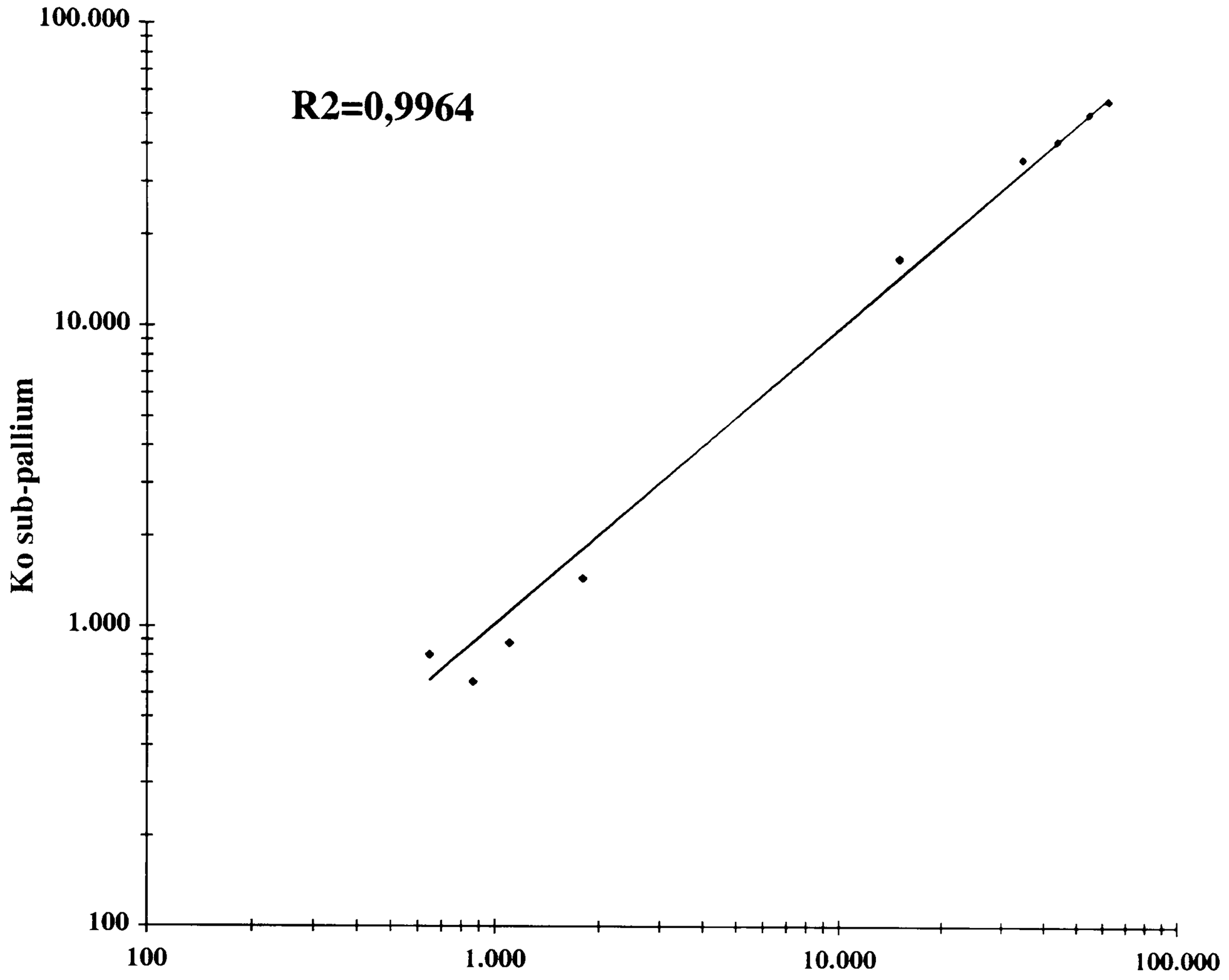
Fig. 16



Scatter plot analysis of Wt sub-pallium versus Dlx1/2 -/- sub-pallium. Average expression levels (arbitrary units) were calculated from two hybridizations (dye swap analysis). Genes that show at least a 4 fold difference are below (down regulated in KO) and above (up-regulated in KO) the dotted lines.

In this experiment the co-efficient of determination was 0.8024, indicating a major difference in the gene expression profile of the two samples. In fact the scatter plot chart (Fig.16) clearly indicates that most of the values lie below the regression line and outside the threshold of 4 fold change, and no *Tess* genes were found to be up regulated in mutant sub-pallium. Since this *Tess* array experiment reported a striking difference in gene expression, it was necessary to verify the hybridization procedure by looking at the behaviour of the yeast intergenic DNA regions present on the array. As expected their values were unchanged in the two channels, thus confirming the consistency of the procedure of hybridization (Fig. 17).

Fig. 17



Scatter plot analysis of Wt sub-pallium versus Dlx1/Dlx2 -/- sub-pallium considering only the yeast intergenic regions (calibrations) expression levels. Average expression levels of ten calibrations were calculated from two hybridizations (dye swap analysis).

A considerable number of clones that in both hybridizations showed a decrease of at least 2 fold, and by t-test analysis were statistically significant ($p<0,02$ in a two tailed analysis), were identified. In order to make a selection of the *Tess* genes differentially expressed, only those with a fold decrease of at least 4 times were further analysed. Table 7 shows the list of the selected *Tess* genes (fold > 4), with a sequence annotation of the clone.

Table 7:

Identity of clones down regulated in Dlx1/2 -/- developing sub pallium

Clone	Fold	Description	Sequence
28.8E	46,51 ± 13,33	Novel	Chr.2
8.12F	25,26 ± 6,60	Novel	Mm.29833
20.6H	8,79 ± 1,98	Novel	Mm.56455
31.3A	7,42 ± 1,67	Novel	AK082430
31.5E	6,44 ± 3,83	Novel	Mm.94021
22.5C	5,17 ± 0,37	Novel	Mm.257560
20.3H	4,85 ± 0,58	Sh3bgrl	Mm.196451
18.6H	4,80 ± 1,32	Novel	Chr.2
18.6D	4,75 ± 0,40	Quaking	Mm.2655
18.7H	4,73 ± 0,46	Nap1L1	Mm.3797
12.5A	4,69 ± 0,83	Novel	Chr.6
9.8C	4,61 ± 1,67	Novel	Chr.16
24.11A	4,60 ± 1,42	Zfp198	Mm.31417
8.10F	4,55 ± 0,36	Novel	Chr.19
23.10E	4,50 ± 1,01	Novel	Mm.111789
19.10B	4,50 ± 0,65	Sig41	Mm.2984
18.12H	4,48 ± 0,45	Xist	Mm.4095
13.10G	4,47 ± 1,03	Novel	Mm.29047
32.9H	4,41 ± 0,36	Novel	Mm.7885
23.9C	4,39 ± 0,95	Cadherin 11	Mm.1571
25.7G	4,38 ± 0,69	Novel	Mm.24023
8.9F	4,37 ± 0,68	M.o.1,2-α-man.	Mm.21596
30.8D	4,32 ± 0,61	Novel	Mm.257158
11.3H	4,31 ± 0,61	Sox21	Mm.70950
13.7A	4,29 ± 0,27	Novel	Chr.4
24.2D	4,29 ± 0,36	Novel	Chr.16
21.1F	4,27 ± 0,30	Novel	Mm.261214
22.12E	4,22 ± 0,57	Novel	Chr.2
26.6D	4,22 ± 0,57	Ncx5l	Mm.257718
26.1H	4,20 ± 0,59	Novel	Mm.260956

Clone	Fold	Description	Sequence
21.1D	4,18 ± 0,83	Formin bp 17	Mm.226935
8.8G	4,18 ± 0,44	Bcl11A-XL	Mm.24020
17.12C	4,15 ± 0,94	Zfp326	Mm.12891
19.3E	4,11 ± 0,61	Foxp2	Mm.101410
12.5D	4,11 ± 0,52	Novel	Mm.79022
25.11A	4,10 ± 0,41	Novel	AI506935
32.7G	4,09 ± 0,70	Novel	Mm.41702
0.6H	4,07 ± 1,57	Smarca5	Mm.133447
28.7F	4,07 ± 0,52	Arhe ras	Mm.46497
11.2D	4,06 ± 0,77	Novel	Mm.259452
8.2B	4,04 ± 0,80	Novel	Chr.8
20.2E	4,03 ± 0,44	Scm-related	Mm.29067
31.7A	4,02 ± 0,44	Novel	Mm.27563
25.5E	4,02 ± 0,47	Novel	Mm.31352
30.5F	4,00 ± 0,39	Zfp62	Mm.16650
32.1H	4,00 ± 0,49	Novel	Mm.257808

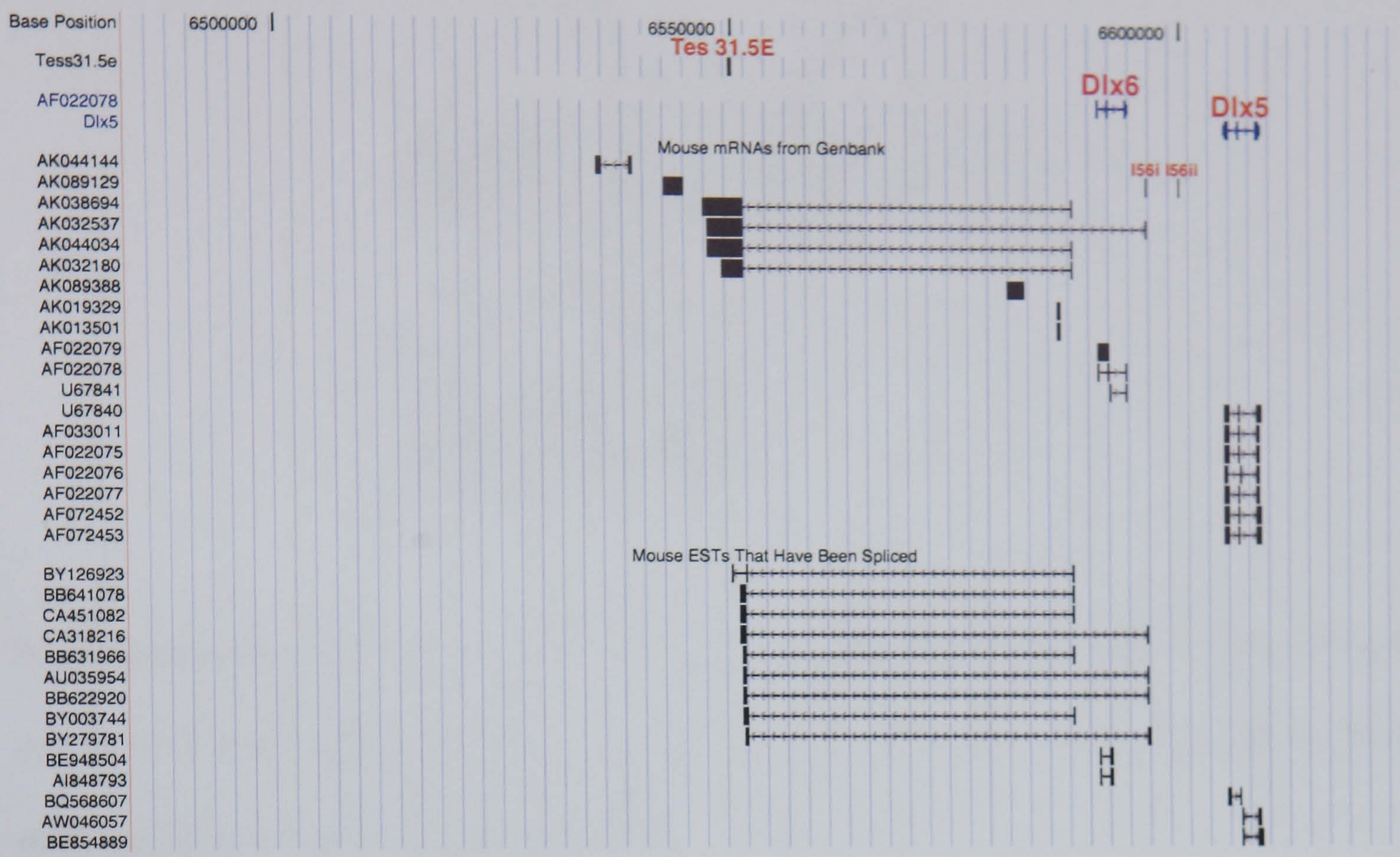
Sequence analysis

The same bioinformatic approach described above was used to identify the genes corresponding to the differentially expressed *Tess* clones. As shown in Table 7, 28 out of 46 genes correspond to novel transcripts. Of the 28 novel genes, 8 had sequence homology with known genes, 11 corresponded to uncharacterised ESTs, and for 9 only a genomic sequence hit could be found. As for the Pax6 and Ngn1/2 mutants, in the double Dlx mutant some *Tess* genes (such as clone 28.8E and 31.5E) were found consistently disregulated (see Discussion). The sequence analysis of clone 28.8E, one of the most promising cDNAs, showed that there were no transcripts or ESTs matching its sequence, and the only information available is the genomic location on chromosome 2 (34400448-34400802). In this region there are few ESTs or transcripts identified, and none of them co-localize with clone 28.8E.

Another interesting clone is 31.5E, which maps on chromosome 6, close to the Dlx5 and Dlx6 genes. This specific region has a highly complex genomic sequence, since there are

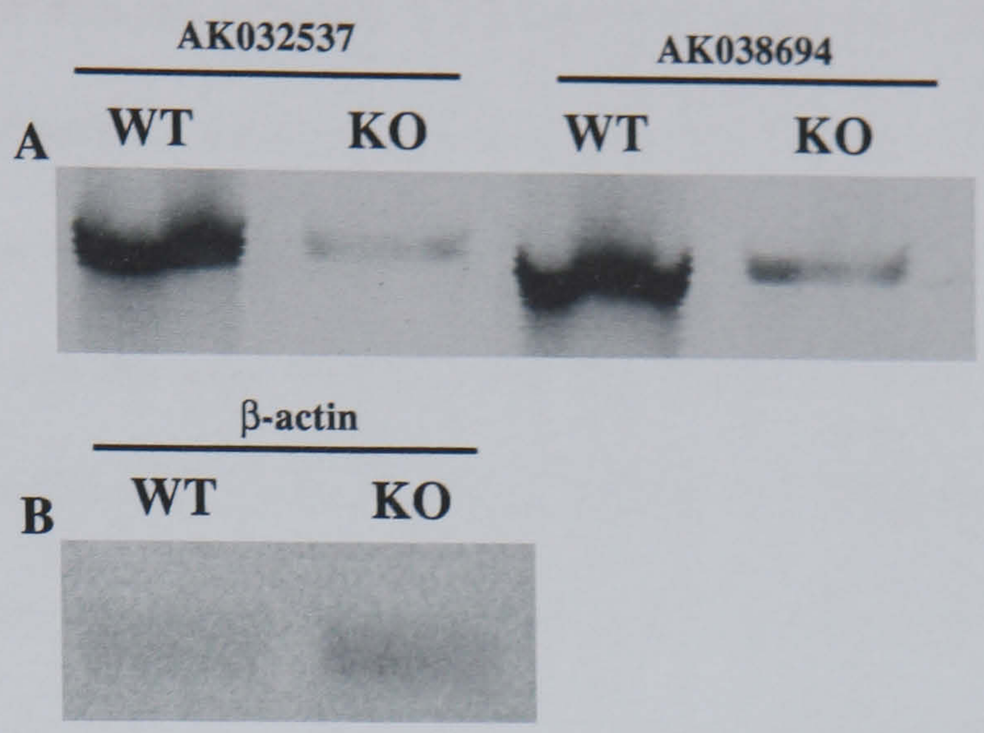
two known genes (Dlx5 and Dlx6), one antisense sequence (antisense to Dlx6) (AF022079) and two Riken transcripts (AK038694 and AK032537). Of these two transcripts one has the 5' end in the intergenic region of Dlx5 and Dlx6 (Fig. 18). In this region there are also highly conserved genomic sequences among different species that are potential sites of action of a number of regulatory factors (Zerucha et al., 2000), among which are the Dlx proteins themselves. Two genomic sequences named I56i and I56ii of 400 and 300 bp are highly similar between zebrafish and mouse (Zerucha et al., 2000), and the Riken transcript AK032537 contains at its 5' end the sequence named I56i (Fig. 18). The two Riken transcripts partially share at the 3' region the sequence of clone 31.5E. In order to verify the expression of the AK032537 and AK038694 transcripts in Wt and Dlx1/Dlx2 mutant mice, RT-PCR has been performed, and as shown in Fig.19, both transcripts were confirmed to be down regulated in the mutant sub-pallium.

Fig. 18



Genomic localization of the Tes clone 31.5E.

Fig. 19



Semi-quantitative RT-PCR analysis on Riken transcripts AK032537 and AK038694 on WT and Dlx1-2 null mutant sub-pallium (KO). In B are shown the levels of β-actin in the WT and Dlx1-2 null mutant sub-pallium (KO).

These two transcripts didn't seem to code for any protein, since using BlastN, BlastX and TBlastX algorithms it was not possible to identify any sequence homologies, or even short conserved protein domains similar to other proteins. These results didn't exclude a potential role of these transcripts. In fact, the observations that both transcripts (AK032537 and AK038694) were in the opposite direction with respect to Dlx6, that their 5' region lay in the intergenic Dlx5/Dlx6 region, and that part of the enhancer I56i was included in 31.5E, lead to the hypothesis of 31.5E being an antisense transcript. In order to verify this hypothesis, bio-informatic tools were used to verify whether this transcript could be considered a true antisense. The specific software application, named AntiHunter (a BLASTN search for antisense transcripts, http://bio.ifom-firc.it/ANTIHUNTER), and facilitating the in-silico identification of potential antisense EST transcripts within a given genomic region of interest (Lavorgna et al., 2004), recognized the presence of 10 canonically spliced antisense matches, all of them belonging to the Riken transcripts AK032537 and AK038694. This suggested the presence of sequences encoding for antisense transcripts in the Dlx5/Dlx6 region, which presumably are under the control of the Dlx1/Dlx2 genes. The presence of an intergenic enhancer for Dlx5/Dlx6, partially overlapping with AK032537, strongly supports this hypothesis. Moreover, the 3' region of both Riken transcripts is located in the 5' untranslated region of Dlx6 where the putative regulatory regions are located.

Regarding the *Tess* clones corresponding, or with similarity, to known genes, the sequence analysis identified six transcription factors, two RNA binding proteins, two kinases, and two SH3 domain containing proteins. One of the transcription factors was Foxp2, a member of the Fox family of winged-helix/forkhead transcription factor genes. This gene is expressed in the postmitotic layer of the ganglionic eminences, and in migratory or post-migratory striatal neurons (Ferland et al., 2003). Since the striatal complex is extremely

affected in Dlx1/Dlx2 mutants (Anderson et al., 1997b), the down regulation of Foxp2 is an indirect validation of the microarray results.

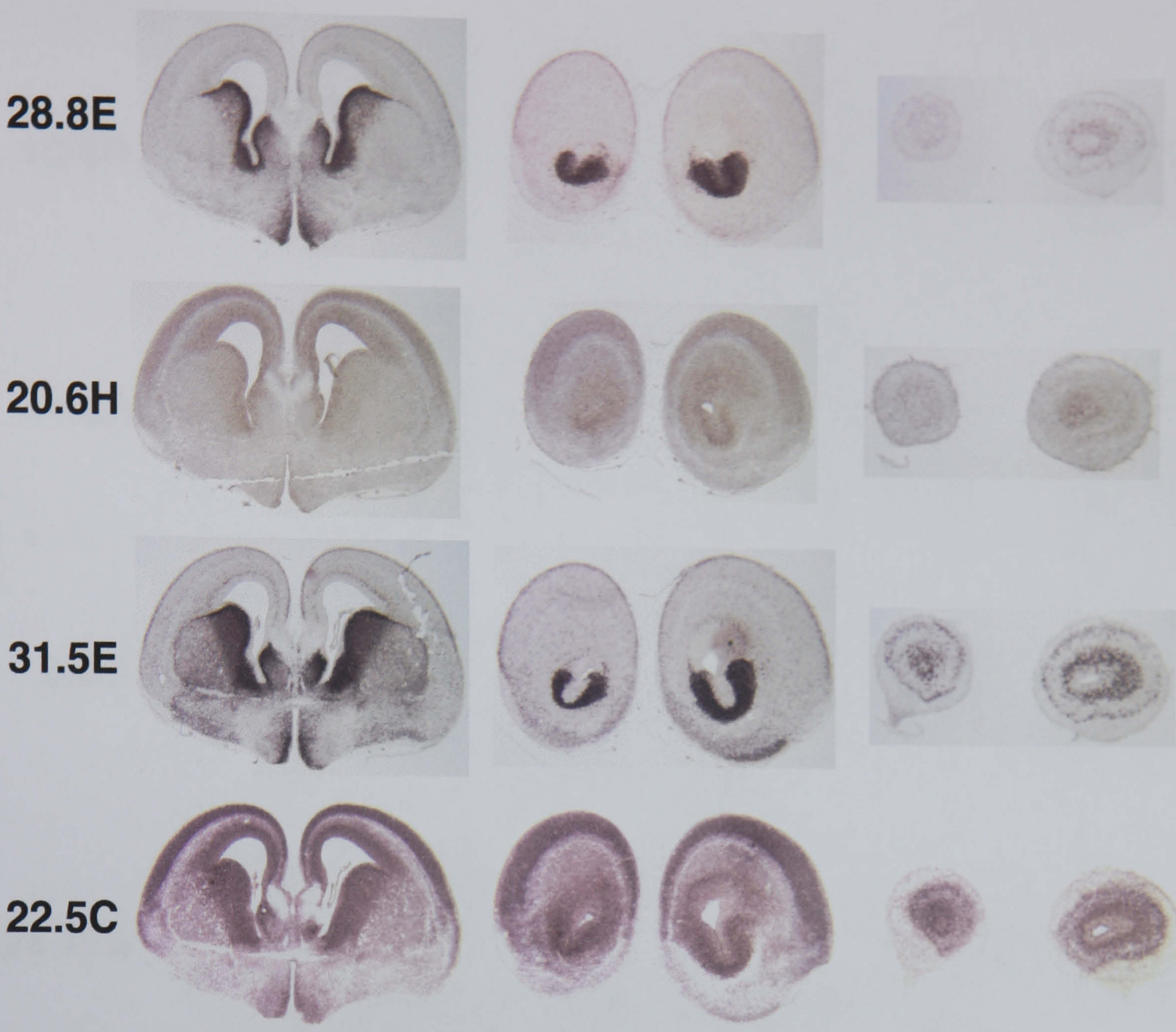
Post analysis validation of microarray experiments

In a microarray experiment, there are many variables that may interfere with the reliability of the data: labelling procedures, scanning process, data quantification and normalization. For these reasons a validation of the microarray data is usually required using an alternative method, at least for the most interesting genes. The validation of data has to provide an independent verification of the gene expression levels, and typically has to be performed on the same RNA samples that were used in the initial array experiments. The commonly used techniques include: semi-quantitative reverse transcription PCR (RT-PCR), real time RT-PCR, northern blot, ribonuclease protection assay, and *in situ* hybridisation. An alternative way to assess the consistency of the microarray data is to compare the array results with information available in the literature and in public expression database.

In order to confirm the microarray data obtained on the mouse mutants RNA *in situ* hybridizations were performed on mouse embryo sections using the *Tess* cDNA clones as templates to generate the probes. This technique allows the detection of the expression patterns of the genes of interest *in vivo*, and for this reason is more informative than quantitative or semi-quantitative RT-PCR. On the other hand RNA *in situ* hybridization is a low-throughput technique, especially in comparison to RT-PCR. Most of the in-situ analysis has been focused on the analysis of the Dlx1/Dlx2 mutants for at least two reasons: (i) this mouse mutant had the highest number of *Tess* clones differentially expressed, and (ii) some of these genes were also dis-regulated in the Pax6 -/- and Ngn1/Ngn2 -/- mutants.

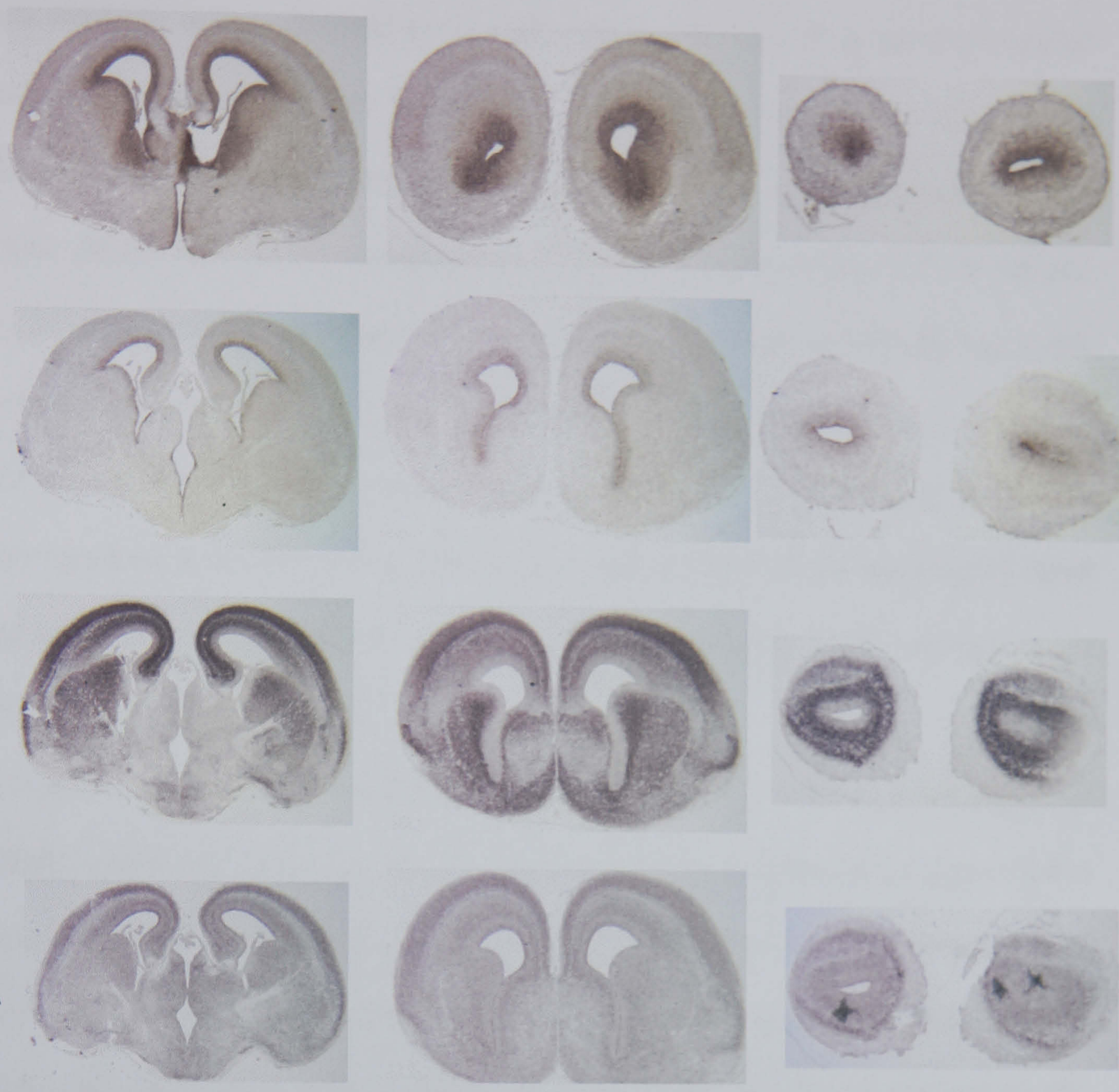
The most differentially expressed *Tess* clones (>4 fold) were first analysed by *in situ* hybridization on WT mouse sections. Fig.20 and 21 show the expression patterns of eight genes with fold changes ranging between 46.51 and 4.18.

Fig. 20



RNA *In situ* hybridizations on WT mouse embryos of the Tes cDNAs down-regulated in Dlx1/Dlx2 double mutant. The images correspond to coronal sections of E14.5 telencephalon hybridized to digoxigenin-labeled RNA probes. The Tes cDNAs were chosen among the most down regulated in the double mutant (Table 7, ranging from 46,51 to 5,17 fold more expressed in the WT embryo).

Fig. 21



RNA *In situ* hybridizations on WT mouse embryos of the Tes cDNAs down-regulated in Dlx1/Dlx2 double mutant. The images correspond to coronal sections of E14.5 telencephalon hybridized to digoxigenin-labeled RNA probes. The Tes cDNAs were chosen among the down-regulated in the double mutant (Table 7, ranging from 4,75 to 4,18 fold more expressed in the WT embryo).

On the basis of their differential expression on the array it was expected that these transcripts would be mainly detected in the ganglionic eminences where the Dlx genes are specifically expressed, and in fact this was the case. For instance, as shown in Fig.20, clones 28.8E, 31.5E and 22.5C were properly expressed in the ventral telencephalon, but while clone 28.8E was exclusively expressed in the proliferative layer of the striatal, pallidal, preoptic and septal neuroepithelium, clone 31.5E was more broadly expressed, and its expression was higher in the proliferative regions than in the differentiating layer. Clone 22.5C was also expressed, even if at lower level than 28.8E and 31.5E, in the ganglionic eminences, but also in the developing cortex. Clone 22.6H even if highly down regulated in the Dlx1/Dlx2 mutant brain, it didn't seem to be specifically expressed in the anlage of the basal ganglia, by *in situ* hybridization.

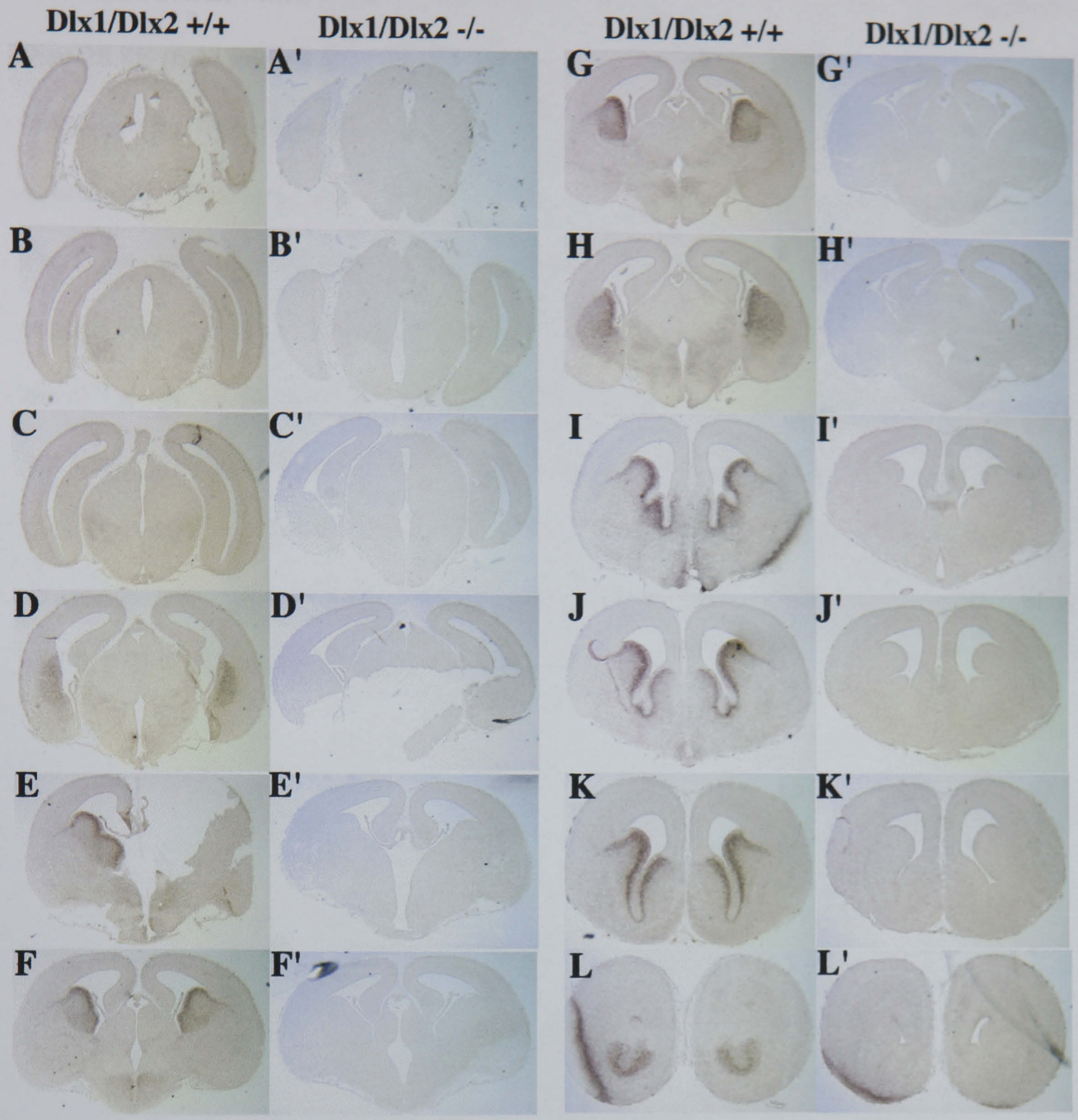
In Fig.21 are reported the expression patterns of another set of *Tess* genes down-regulated in Dlx1/Dlx2 mutants. For these genes the fold change was lower than of those reported in Fig.20, but nevertheless their expression patterns were of great interest. For instance, clone 18.6D was specifically expressed throughout the VZ of the developing telencephalon, suggesting a possible role in the proliferative cell compartment. Conversely clone 8.8G, was mainly expressed in the differentiated cells of both dorsal and ventral telencephalon. A similar regional expression pattern, but restricted to the VZ, was found for clone 11.3H. Clone 24.11A was specifically expressed, even if at low levels, in the telencephalon.

Next step was to determine whether the down-regulated genes found by microarray hybridization, and with the most interestingly expression pattern by RNA in situ hybridization, were also differentially regulated *in vivo* in the mutant telencephalon. The analysis in the mouse mutant telencephalon was mostly restricted to clones 28.8E and 31.5E. The reasons these clones were chosen first was that they were (i) novel genes (see sequence analysis), (ii) highly down-regulated (Table 7), and (iii) specifically expressed in

the WT sub-pallium (Fig.20). *In situ* hybridizations were performed using serial sections of the entire WT and Dlx1/Dlx2 double mutant embryonic telencephalons.

Fig.22 shows the i*n situ* hybridization analysis for clone 28.8E. In the Dlx double mutant brain its expression is completely lost throughout the telencephalon, from the more caudal (Fig.22A') to the more rostral (Fig.22L') territories, while the expression in the WT telencephalon is clearly detectable in the VZ and SVZ of both ganglionic eminences, and also in the pallial/sub-pallial boundary (PSPB) (or corticostriatal boundary). These data validate the gene chip analysis, and confirmed that the *Tess* clone 28.8E, the more highly down-regulated gene (Table 7) in the *Tess* array, is a strong candidate for being regulated by the Dlx genes.

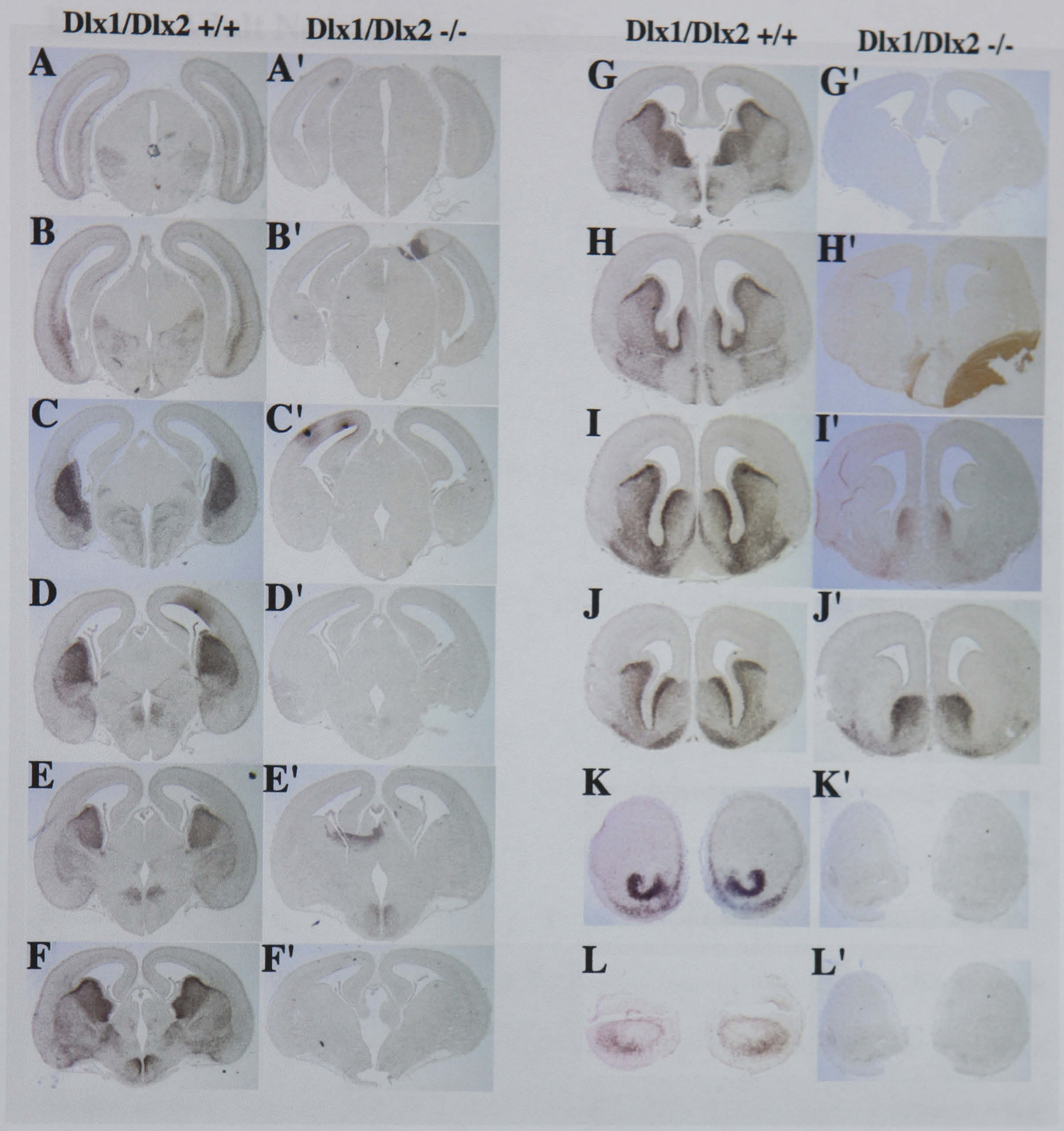
Fig. 22



RNA *In situ* hybridizations on WT (A-L) and null mutant (A'-L') embryonic telencephalons of the Tes clone 28.8. The images correspond to coronal sections of E14.5 telencephalon hybridized to digoxigenin-labeled RNA probes.

Tess clone 31.5E, which in the microarray hybridizations showed a lower down-regulation than 28.8E (6.44 and 46.51, respectively), was also examined for its tissue expression by RNA in situ hybridization. As expected by the array results, also by RNA in situ hybridization this clone was confirmed to be not completely down regulated. As shown in Fig.23, it was still possible to detect 31.5E expression in some regions (for example the septum in Fig. 23J') of the mutant telencephalon. The expression pattern of this clone in the WT brain was slightly different from that of 28.8E, since both were expressed in SVZ, but while 31.5E was also detectable in the differentiating layer, clone 28.8E was expressed, even if at low levels, in the VZ. As shown in Fig. 23J', paradoxically in the septum 31.5E expression was not only down regulated in the mutant, but it seemed to be higher than in the WT (Fig. 23J), in particular in the medial septum.

Fig. 23



RNA *In situ* hybridizations on WT (A-L) and null mutant (A'-L') embryonic telencephalons of the Tes clone 31.5E. The images correspond to coronal sections of E14.5 telencephalon hybridized to digoxigenin-labeled RNA probes.

Results-Adult Neural Stem Cells

Experimental approach

Serum-free CNS cell cultures represent a selective system in which most primary differentiated neural cells are eliminated while the undifferentiated stem cells enter into an active proliferative state. Four conditions are fundamental in the culture system: (i) low cell density, (ii) absence of serum, (iii) addition of the appropriate growth factors, and (iv) absence of a strong cell adhesion substrate. Under these conditions, within 2-3 days, a very small fraction of undifferentiated precursor cells begin to proliferate. The round cell clusters of proliferating cells, named "neurospheres", eventually lift-off the substrate and float in suspension.

An important point has to be carefully evaluated when microarray experiments are performed on neural stem cells: the so called "gene expression" profile of neural stem cells varies depending on the source of RNA that is used to perform the microarray hybridization. In fact, some studies have been performed already (Geschwind et al., 2001; Karsten et al., 2003; Luo et al., 2002) using entire neurospheres as a source of RNA, and is important to know that most of the cells present in the spheres are not stem cells, but only a fraction of them (ranging from 10 to 50% of the total cells) retain stem cell features, while the others undergo spontaneous differentiation. In sum, a neurosphere is a mixture of stem cells, differentiating progenitors, and terminally differentiated neurons and glia; thus the RNA obtained from the neurospheres is representative of this cellular heterogeneity.

In setting up such experiments, one has to keep in mind that the ultimate goal is to obtain a quantitative analysis of gene expression, hence it is critical to obtain RNA from homogeneous cell populations: allowing an accurate comparative quantitative analysis of the transcripts.

For these reasons, in setting up my study on gene expression profiling using the *Tess* array, I decided to use, as a starting material, a pure population of neural stem cells (named as "doublets") collected 24 hours after the dissociation of the neurospheres (in the presence of Fgf2 and Egf). This cell population was actively proliferating and highly enriched for neural stem cells, allowing the collection of more homogeneous stem cell populations than from the neurospheres.

Moreover, in all of the already published studies, researchers have compared only two cell populations: the stem/progenitor cells and a more differentiated population. Since I was interested in the identification of the *Tess* genes involved in both stem cell proliferation and differentiation, I decided to compare the gene expression profiles of these cells at three different moments, and thus I also included the analysis of gene expression in the intermediate committed progenitor cells.

In order to make clear the distinction between stem cells and transiently dividing progenitors it has to be pointed out that the definition of CNS *stem cells* is applied to neural precursors that show extensive self-renewing capacity, and can be propagated for months displaying a steady capacity to generate neurons, astrocytes and oligodendrocytes (*multipotential*). The term *progenitor cells* is used to indicate undifferentiated cells possessing limited proliferative capacity and more restricted developmental potential.

In order to carry out this comprehensive analysis during neural stem cells differentiation, three different competitive hybridizations were performed, and each of them was repeated three times (Materials and Methods): neural stem cells (NSCs) (collected as described above) versus progenitor cells, NSCs versus differentiated cells, and the progenitor cells versus differentiated cells. The progenitor cells were grown for 3 days in the presence of Egf alone, whereas the differentiated cells were left to differentiate for 5 days in 1% serum (Table 8).

Table 8

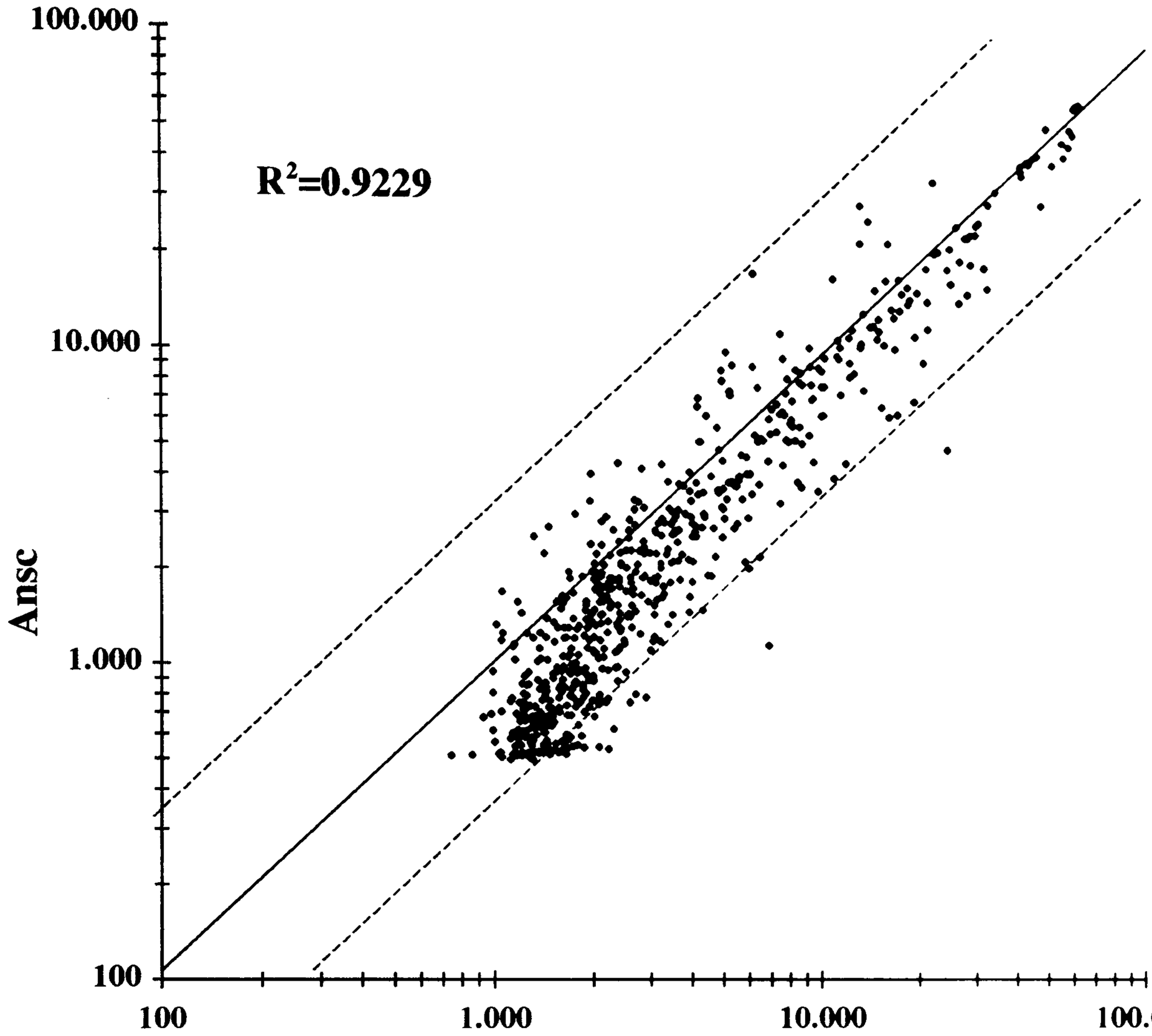
Cells	NSC	PROG	DIFF
Time	24h →	3 days →	3days →
Mitogens	FGF + EGF	FGF	No growth factors
Medium	No serum	No serum	1% serum

In these experiments, in order to avoid false positives, only the *Tess* genes that in the three comparisons (Nsc vs Prog; Nsc vs Diff; Prog vs Diff) showed at least two fold difference were considered for further analysis (even if in some cases the threshold had to be lowered to 1.5, see below).

Neural stem cells vs. progenitors cells

In this experiment we used neural stem cells grown for 24h in the presence of Fgf2-Egf, and progenitor cells grown for 3 days in the presence of Fgf2 alone. The microarray hybridizations using the *Tess* chip showed that these two populations have a similar gene expression profile (as shown in Fig.24 the R squared value = 0,9229), but nonetheless some *Tess* genes were truly differentially expressed.

Fig. 24



Scatter plot analysis of adult neural stem cells (Ansc) versus progenitor cells (Prog). Average expression levels (arbitrary units) were calculated from two hybridizations (dye swap analysis). Genes that show at least a 2 fold difference are below (up-regulated in progenitor cells) and above (up regulated in neural stem cells) the dotted lines. The genes that are between the two dotted lines are considered equally expressed.

In particular, only a few genes were specifically expressed in the neural stem cells, while more genes were preferentially expressed in the progenitor cells. PCNA, a marker of cell proliferation and an internal control for the coherence of the experiment, was consistently found to be expressed more in neural stem cells (the fold change was 2.97 ± 0.35). Table 9 shows the list of *Tess* clones differentially expressed in neural stem cells (A) and in neural progenitor cells (B).

Table 9

A. Clones expressed more in neural stem cells

Clone	Fold change	Description	Sequence
21.12E	2,4±0,26	Cyclin D2	NM_009829
7.3G	1,91±0,12	Pcmt1	NM_008786
23.3D	1,9±0,24	Novel	Mm.45361
29.5G	1,81±0,31	Hmgb2	NM_008252

B. Clones expressed more in progenitors

Clone	Fold change	Description	Sequence
32.4D	6,09±1,32	Connexin 43	NM_010288
21.5C	2,68±0,19	Sox4	NM_009238
30.2D	2,55±0,14	Polybromo1	AK009120
8.12C	2,47±0,58	Vrk1	NM_011705
26.3G	2,22±0,24	Reticulocalbin	NM_009037
31.5A	2,1±0,22	Prolactin like M	Mm.200292
9.4G	2±0,05	Slc16a4	BC026596

Four *Tess* clones were found to be preferentially expressed in neural stem cells, and even if their values were around the two-fold difference threshold, they were consistently up regulated in the different experiments.

Tess 29.5G, one of the clones more expressed in the neural stem cell population, turned out to correspond to the Hmgb2 gene (Javaherian et al., 1978), a member of the HMGB protein family, which includes the ubiquitous Hmgb1 and the embryo specific Hmgb3.

Tess 23.3D (UniGene cluster Mm.45361) may instead encode for a novel protein, with a weak protein homology (30.42%) with a WD repeat domain protein, the nuclear protein

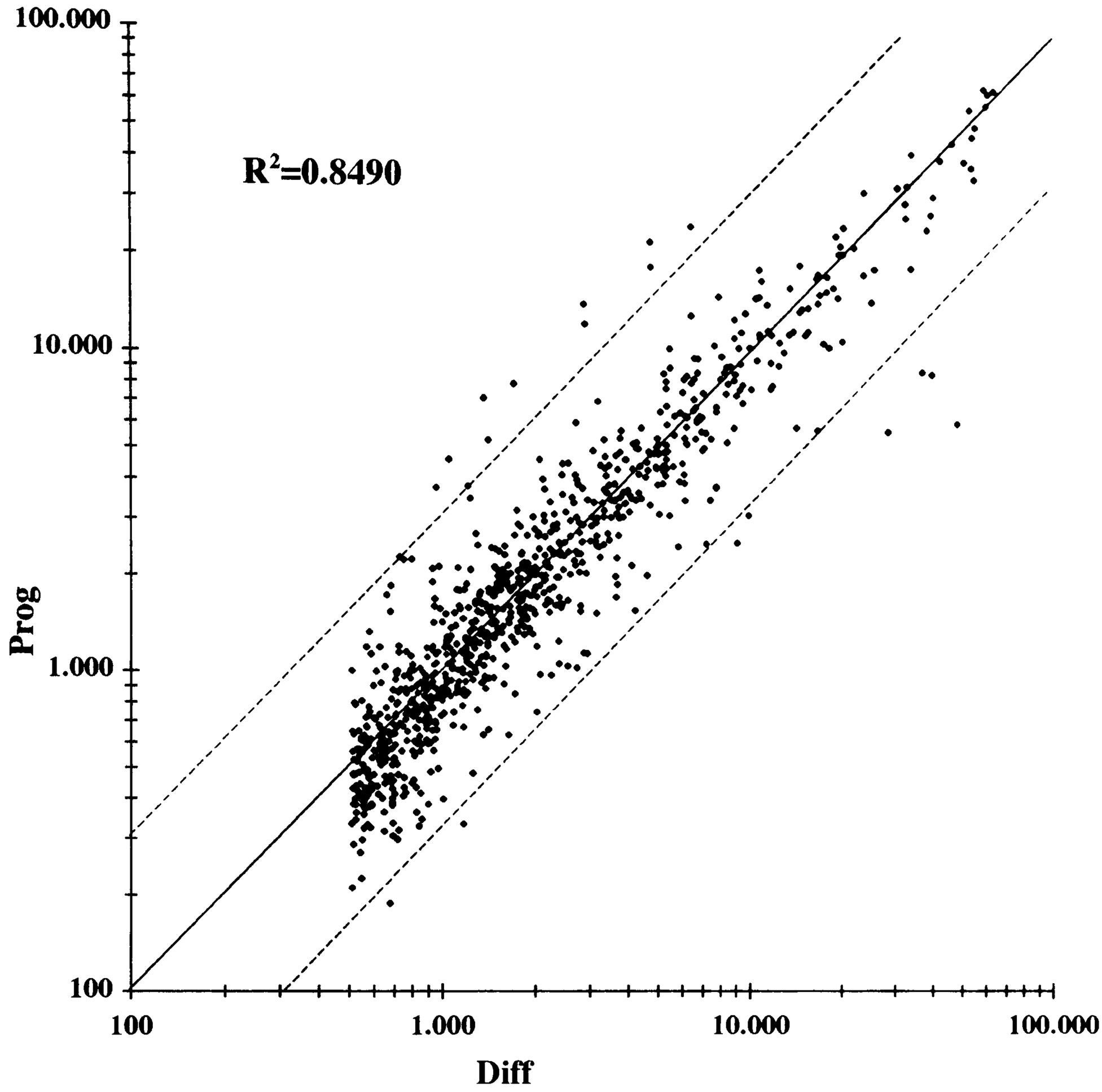
Ytm1. Two other genes were more expressed in the neural stem cells: clone 7.3G, corresponding to Pcmt1 (protein-L-isoaspartate O-methyltransferase 1), a protein repair enzyme which acts on proteins with abnormal accumulation of aspartyl residues during cell aging; and 21.12E, corresponding to cyclin D2.

Seven genes were differentially expressed at least two times more in the progenitors cell respect to the Nsc. Among these there was *Tess* 21.5C that corresponds to Sox4, a gene known to play a critical role in embryonic neural progenitors (Cheung et al., 2000), and *Tess* 31.5A corresponding to the Prolactin–like protein M, which has a consensus site for N-linked glycosylation.

Progenitor cells vs. differentiated cells

In this experiment, neural progenitor cells were compared to differentiated neural cells (obtained as described above). The progenitor cells usually differentiate in culture into neurons (35%), oligodendrocytes (10%) and astrocytes (55%) (Gritti et al., 1996). As shown in the scatter plot of Fig.25, the R squared value was 0,8490, indicating that there were more differences in gene expression between the two populations than between neural stem cells and neural progenitors (R^2= 0,9229).

Fig. 25



Scatter plot analysis of progenitor cells (Prog) versus differentiated cells (Prog). Average expression levels (arbitrary units) were calculated from two hybridizations (dye swap analysis). Genes that show at least a 2 fold difference are below (up-regulated in differentiated cells) and above (up regulated in progenitor cells) the dotted lines. The genes that are between the two dotted lines are considered equally expressed.

Table 10 shows the list of *Tess* cDNAs that were expressed more in neural progenitors (A) and in differentiated cells (B).

Table 10

A. *Tess* clones preferentially expressed in progenitors

Clone	Fold change	Description	Sequence
18.11A	5,6±1,53	Foxg1/Bf1	NM_008241
12.6B	5,01±1,22	Mest/Peg1	NM_008590
18.11E	1,98±0,14	Tubulin beta IV	NM_009451
18.9A	1,77±0,32	Rad23b	NM_009011

B. *Tess* clones preferentially expressed in differentiated cells

Clone	Fold change	Description	Sequence
32.4D	6,09±1,32	Connexin 43	NM_010288
30.12E	3,4±0,61	Novel	Mm.136892
28.1B	3,28±0,56	Calponin 3 acidic	NM_028044
22.10C	2,93±0,29	Zfp 131	BC048839
9.4G	2,9±0,62	Slc16a4	BC026596
26.3G	2,22±0,24	Reticulocalbin	NM_009037

The clone *Tess* 18.11A showed the highest fold change in progenitors, and corresponds to Foxg1/Bf1, a winged helix gene with a pleiotropic role in brain development. It has been shown that telencephalic progenitors lacking Foxg1/Bf1 differentiate into neurons prematurely (Hanashima et al., 2002). A similar difference in expression was observed for clone 12.6B, corresponding to Mest/Peg1, an imprinted gene paternally expressed and playing a fundamental role in embryonic development (Lefebvre et al., 1998). To date this gene has been studied for its involvement in heart (King et al., 2002) and kidney (Kanwar et al., 2002) development, but not for its role in telencephalic development and neural stem cell biology. Clone 18.9A corresponded to Rad23b, a DNA repair enzyme (UV excision repair protein), and *Tess* 18.11E corresponded to tubulin-beta IV.

In the differentiated cells the clone with the highest fold change was *Tess* 32.4D, and it is worth nothing that this clone was also the most up-regulated in progenitor cells compared to neural stem cells. It corresponds to Connexin 43 (gap junction membrane channel

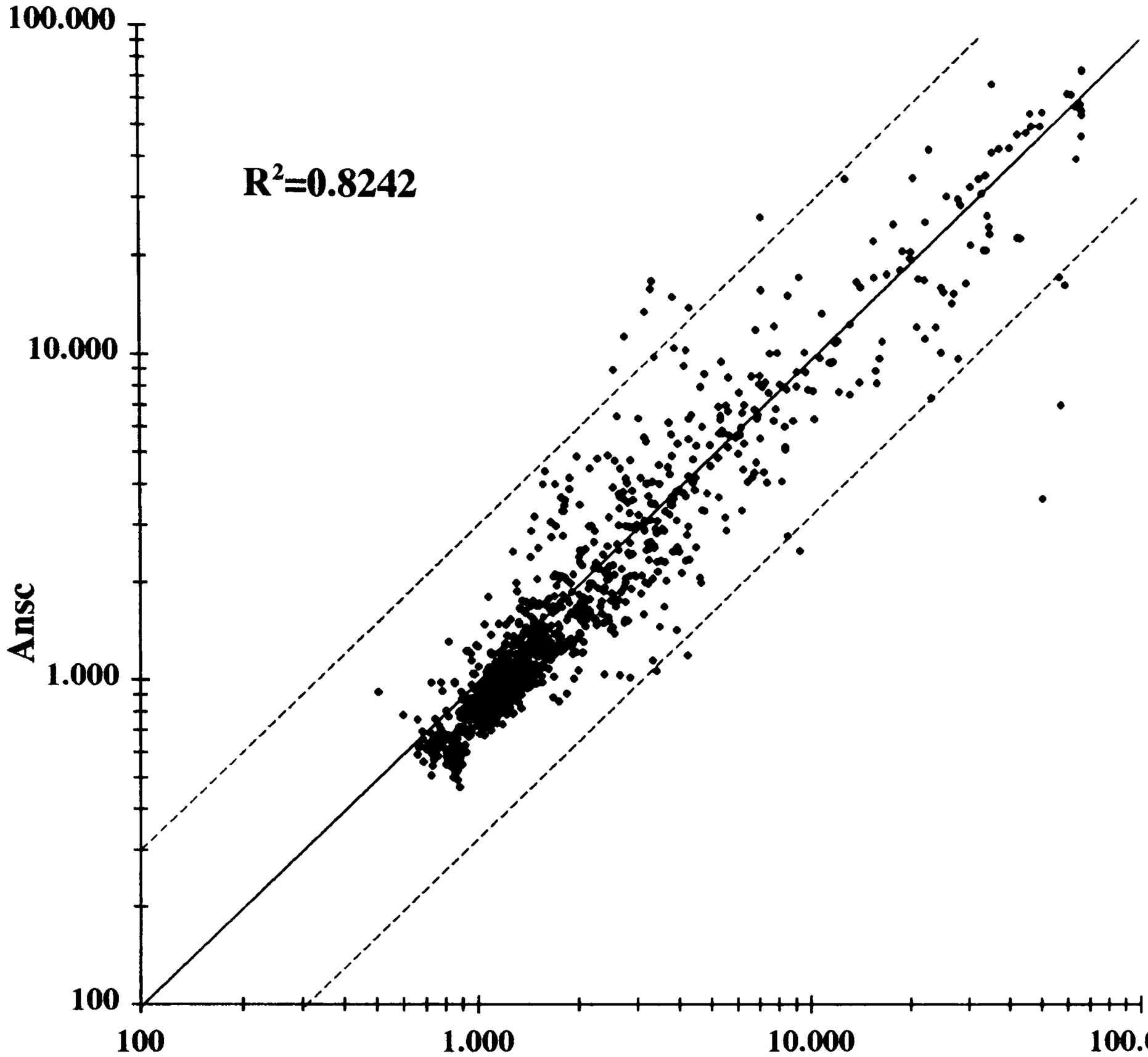
protein alpha 1), a protein with gap-junction forming channel activity. There were two other clones already present in the neural stem cells-progenitors comparison with an increased expression in progenitors, which were also expressed more in differentiated cells than in the progenitors: *Tess* 26.3G, corresponding to Reticulocalbin, and 9.4G, corresponding to a putative sugar transporter.

Tess 30.12E (UniGene cluster Mm.136892) was 3,4 fold more expressed in differentiated cells, and corresponds to a hypothetical protein weakly similar to NCK-Associated Protein NAP5. Clone 22.10C turned out to be the zinc finger protein Zfp131.

Neural stem cells vs. differentiated cells

As expected, these two cell populations were characterized by a significant difference in gene expression profile. As shown in Fig.26, the R squared value was 0.8242.

Fig. 26



Scatter plot analysis of adult neural stem cells (Ansc) versus differentiated cells (Diff). Average expression levels (arbitrary units) were calculated from two hybridizations (dye swap analysis). Genes that show at least a 2 fold difference are below (up-regulated in differentiated cells) and above (up regulated in neural stem cells) the dotted lines. The genes that are between the two dotted lines are considered equally expressed

Table 11 shows the list of the *Tess* genes that are expressed more in neural stem (A) or in differentiated cells (B).

Table 11

A. *Tess* clones expressed more in neural stem cells

Clone	Fold change	Description	Sequence
12.6B	6,25±1,7	Mest/Peg1	NM_008590
18.11A	5,2±2,22	Foxg1/Bf1	
13.6H	3,72±0,72	Sox11	
29.5G	3,7±1,01	Hmgb2	NM_008252
18.9A	2,98±0,36	Rad23b	NM_009011
9.5A	2,91±0,32	Novel	No hit
27.6A	2,67±0,68	Novel	No hit
13.6C	2,46±0,45	Stag2	NM_021465
21.12E	2,96±0,16	Myotubularin	NM_016985

B. *Tess* clones expressed more in differentiated cells

Clone	Fold change	Description	Sequence
32.4D	14,43±3,09	Connexin 43	NM_010288
26.3G	4,59±2,58	Reticulocalbin	NM_009037
9.4G	3,06±0,25	Slc16a4	BC026596
28.1B	3,03±0,25	Calponin 3 acidic	NM_028044
30.12E	2,61±0,36	Novel	Mm.136892
8.12C	2,27±0,29	Vrk1	NM_011705

Clone 12.6B (Mest/Peg1), clone 18.11A (Foxg1/Bf1), and clone 18.9A (Rad23b) were among the most up-regulated genes in neural stem cells and also in the progenitor cells when compared to the differentiated cells. Clone 13.6H corresponds to Sox11, in agreement with the observation that Sox proteins play a relevant role in stem cell biology (Cheung et al., 2000). Clone 21.12E (Myotubularin) and 29.5G (Hmgb2), were among the most expressed genes of the *Tess* array in neural stem cells when compared to the progenitors (Table 9), and in this analysis are also definitively expressed more highly in neural stem cells compared to the differentiated cells supporting again their probable role in stem cell biology.

In this analysis, new *Tess* clones were found more highly expressed in neural stem cells, and were not identified in the previous analysis. Two of them correspond to novel genes. *Tess* 9.5A and *Tess* 27.6A, which both had no hits in the EST database, but their sequences corresponded only to genomic sequences in the Mus Musculus genomic database. Another clone, 13.6C, corresponded to Stag2 (stromal antigen 2), a gene with a role in cell cycle and chromosome segregation.

Tess 32.4D, corresponding to Connexin 43, was the clone with the highest difference in expression level. Since in all three analyses it was consistently up-regulated in the more committed population, and it can be viewed as a marker of differentiation. The same is also true for the *Tess* clones 26.3G (Reticulocalbin), 9.4G (Slc16a4), 28.1B (Calponin 3), 30.12E (novel) and 8.12C (novel).

Clustering of the data

In order to unravel the molecular network underling neural stem cell differentiation, a cluster analysis of the *Tess* clones found differentially expressed in the process of differentiation has been performed. A cluster analysis of the gene expression data acquired at different points of an experimental timeframe is critical in order to exploit the power of microarray technology. The cluster analysis algorithm, based on Euclidean distance measurements, has been used to identify specific profiles of gene expression within the set of *Tess* genes. The rationale behind such an analysis is that gene expression is the result of coordinated activation of genes that are linked together because they belong to similar functional categories or pathways. The clustering process generates a sorted representation of expression profiles enabling the investigator to identify sets of co-regulated genes.

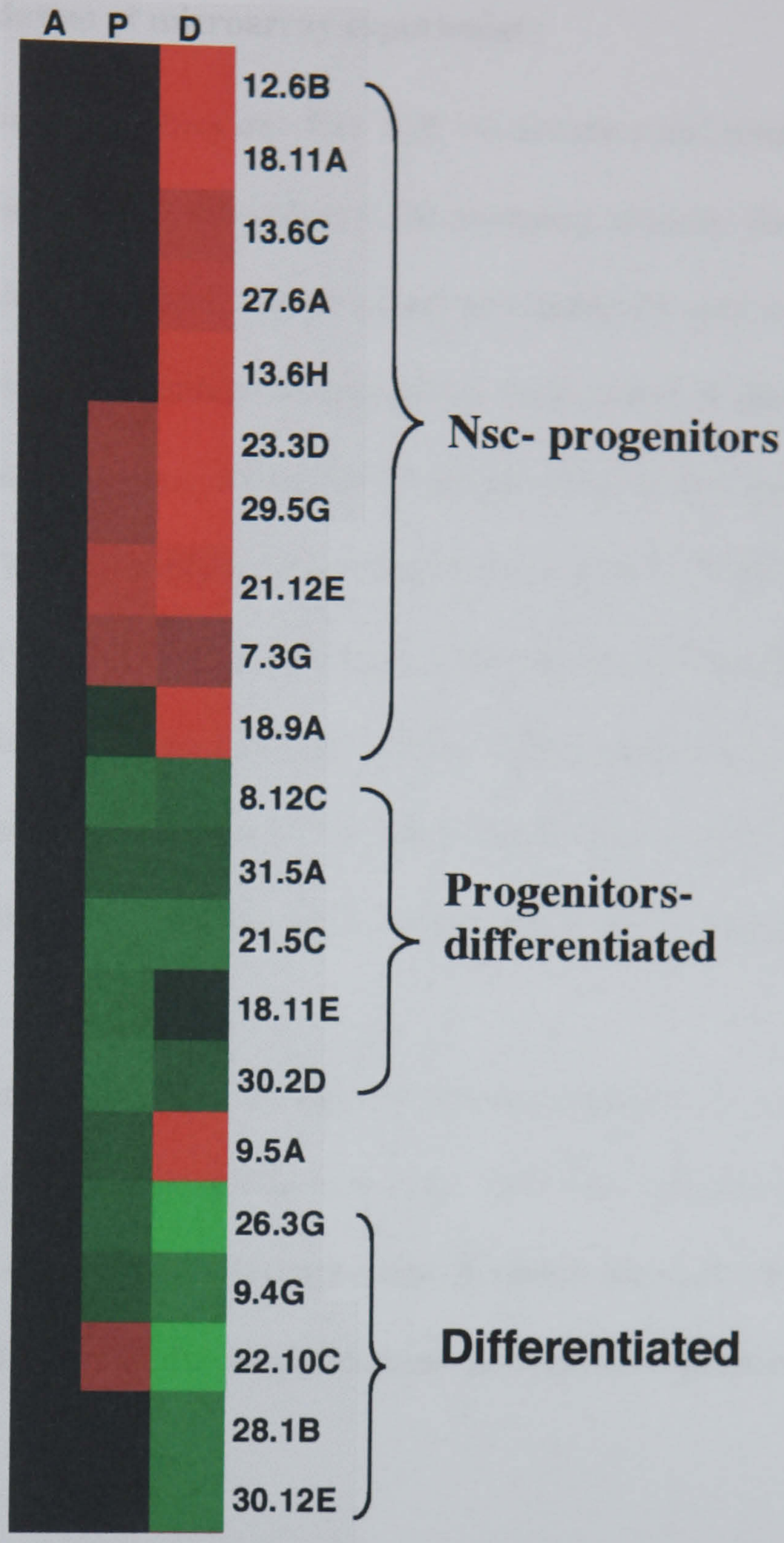
This analysis was performed considering the three cellular populations (NSC, PC, DC) as distinct points in the temporal acquisition of a differentiated phenotype: neural stem cells $\Rightarrow$ progenitors $\Rightarrow$ differentiated cells. To perform the computational analysis an arbitrary value of 1 has been assigned to the intensity of all the spots detected using the neural stem

cells population, and for the other two populations the variation of intensity (in terms of fold change) with respect to this starting point was analysed. Table 12 shows the numerical clustering of the *Tess* clones, whereas Fig. 27 shows the Caged (see Materials and Methods) graphic analysis of the three sets of microarray experiments. In the clustering process it was possible to identify clusters (or groups) of genes that are clearly down or up regulated upon differentiation.

Table 12

clone	nsc	prog	diff
32.4D (Connexin 43)	1	6,09	14,43
22.10C (Novel)	1	-1,85	5,42
26.3G (Reticulocalbin)	1	2,22	4,59
9.4G (Slc16a4)	1	2	3,06
28.1B (Calponin 3)	1	1	3,03
30.12E (Novel)	1	1	3
21.5C (Sox4)	1	2,68	2,68
8.12C (Vrk1)	1	2,47	2,27
30.2D (Polybromo 1)	1	2,55	2,04
31.5A (Prolactin like M)	1	2,1	2
18.11E (Tubulin beta IV)	1	2,5	1,26
7.3G (Pcmt1)	1	-1,91	-1,72
13.6C (Stag2)	1	1	-2,46
27.6A (Novel)	1	1	-2,7
9.5A (Novel)	1	2,1	-2,91
18.9A (Rad23b)	1	1,42	-2,98
21.12E (Cyclin D2)	1	-2,4	-3,03
13.6H (Sox11)	1	1	-3,31
29.5G (Hmgb2)	1	-1,81	-3,7
23.3D (Novel)	1	-1,9	-4,76
18.11A (Foxg1)	1	1	-5,4
12.6B (Mest/Peg1)	1	1	-6,53

Fig. 27



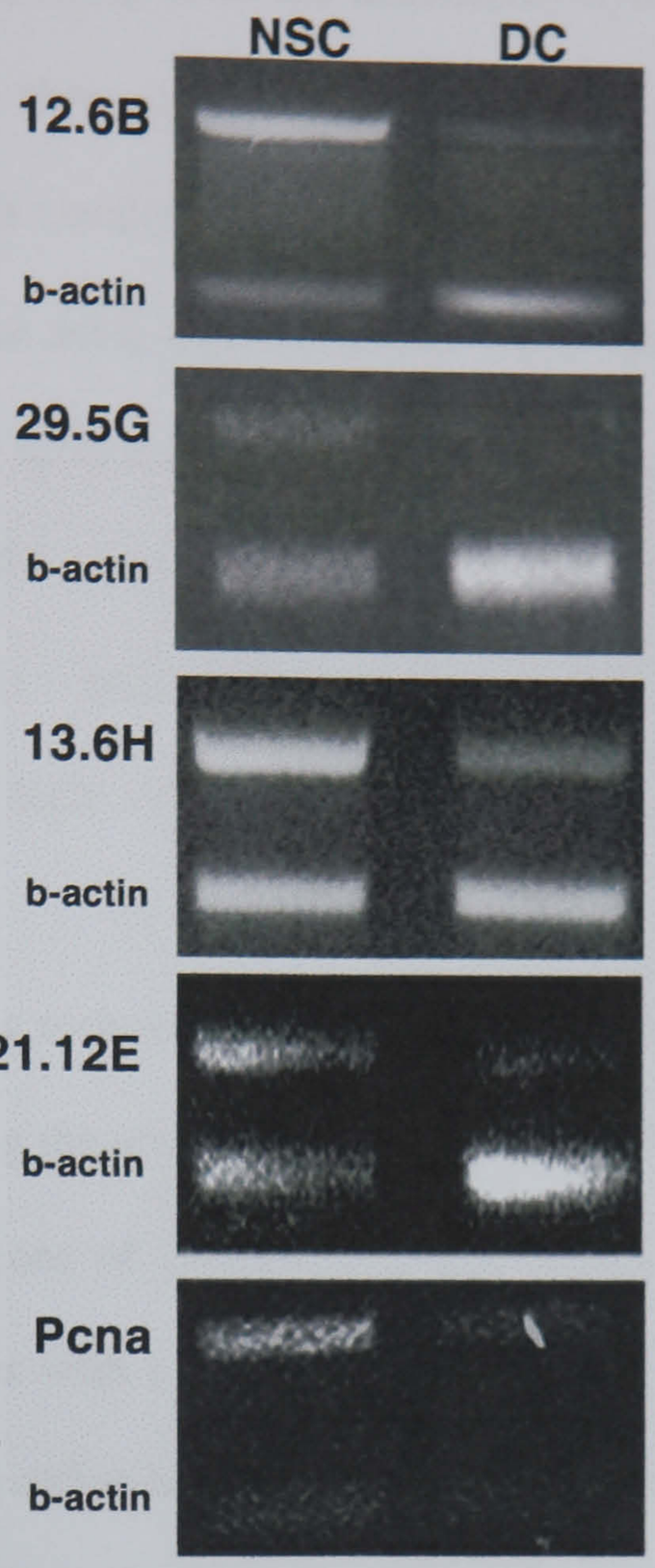
Cluster analysis of the spots identified as differentially expressed in the three populations. Expression values higher or lower than 1 are reported in two different colors: green and red, respectively. The intensity of each color is proportional to the fold-differencee of each value from the neural stem cell population. Abbreviations: A, adult neural stem cells; P, progenitor cells; D, differentiated cells.

Post analysis follow-up and validation of microarray experiments

In using microarray technology, many variables interfere with the accuracy and reliability of the data that are generated: the labelling procedures, the scanning process, the data quantification and normalization. For this reason it is necessary to validate the data with an alternative method, which provides independent, experimental verification of the gene expression levels, and typically uses the same original RNA samples that were employed in the initial array experiments. The commonly used techniques include semi-quantitative reverse transcription PCR (RT-PCR), real time RT-PCR, northern blot, ribonuclease protection assay, and *in situ* hybridization. An alternative, or complementary approach to confirm the microarray data, is to perform a sort of "*in silico*" analysis, in which array results are compared with the information available in literature and in public expression database.

Semi-quantitative RT-PCR was used to confirm the data of the most relevant *Tess* genes with a fold change in gene expression superior to two (Fig. 28). The validation was focused mostly on the transcripts with a preferential expression in neural stem cells. PCNA expression was tested as control, and all the amplifications performed confirmed the microarray analysis.

Fig. 28



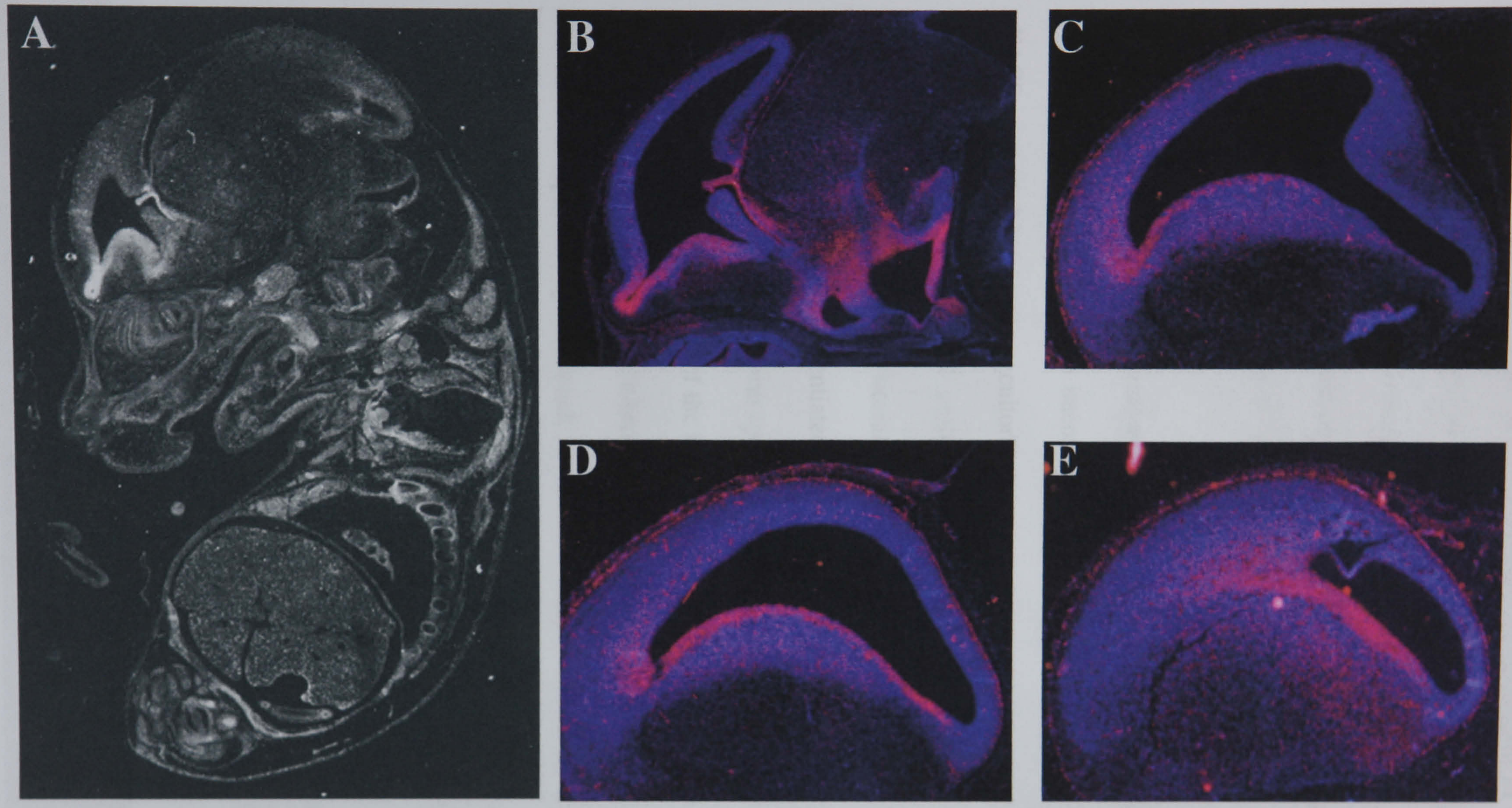
Semi-quantitative RT-PCR analysis on Tes clones up-regulated in neural stem cells respect to differentiated cells. In each panel, the upper band corresponds to the clone of interest. The lower band corresponds to the β-actin internal control.

Such confirmation was not surprising since the microarray hybridizations were carried out as accurately as possible: (i) three different hybridizations for each comparison were performed (a dye swap analysis usually requires only two hybridizations); (ii) two different sets of RNA preparations were used, and the results were comparable; and (iii) only the clones that were consistently differentially expressed in at least two comparisons were clustered. This approach had the aim to exclude false positives in the screening, even if it led to the exclusion of *Tess* genes (for example between neural stem cells and differentiated cells) because their values were not consistent in at least two different comparisons.

RNA *in situ* hybridization is a technique that detects gene expression directly on tissue, and hence is used for validating the array data but also for gaining insights into the putative biological function of the genes of interest. For this reason in situ hybridization was performed for the *Tess* clones with a known role in development, and with the highest difference in expression in the differentiation experiments of neural stem cells: *Tess* 12.6B (Mest/Peg1), *Tess* 29.5G (Hmgb2), *Tess* 23.3D (novel), *Tess* 7.3G (Pcmt1) and *Tess* 31.5A (Prolactin like M). For the Mest/Peg1 gene, a detailed in situ hybridization was carried out, due to its high fold change, its characteristics of imprinted gene, and because it was never correlated to stem cell biology.

In situ hybridizations were performed using the same clones arrayed on the chip as probes. At 14.5 days (Fig.29), the *in situ* hybridisation shows a very fine and specific expression of Mest/Peg1 in the ventricular zone of the basal telencephalon, in the choroid plexus, in the olfactory bulb and in the septum. These data correlate well with its up regulation in stem cells since the ventricular-subventricular zones are the sites where stem/progenitor cells reside and proliferate. For this gene RT-PCR, in *situ* hybridizations and microarray analysis indicate that Mest/Peg1 is an important player in stem/progenitors cell biology.

Fig. 29



In situ expression pattern of Mest/Peg1. (A) Image from sagittal section of whole embryo showing the specific localization of Mest/Peg1 in the forebrain proliferative zone. (B-E) high magnification of forebrain expression on sagittal serial sections.

Several papers have been published on Mest/Peg1 expression during development. It is first detected in the embryonic and extra-embryonic mesoderm of gastrulating mouse embryos, while subsequently is expressed in all mesodermal derivates, and specific regions of the developing brain (Lefebvre et al., 1998). During development, only the paternally inherited Mest allele is expressed, and the function of the MEST protein is still unknown, although similarities with the α/β-hydrolase fold family suggests an enzymatic role (see discussion).

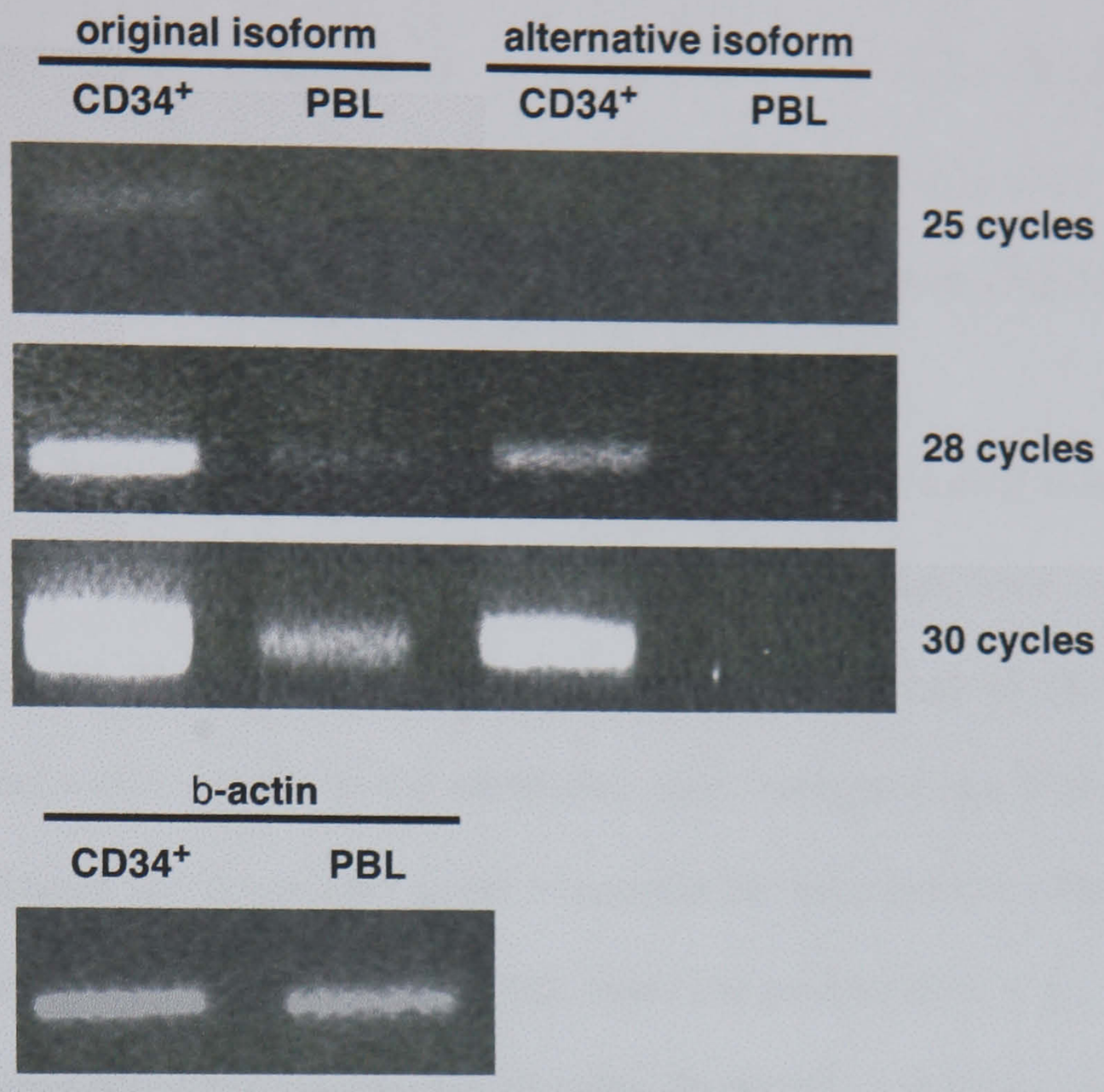
Because of its expression in neural stem cells, and in all mesodermal derivates, an analysis was performed also in human haematopoietic stem cells, in particular in bone marrow FACS-sorted $CD34^+$ stem/progenitor cells.

For this purpose human $CD34^+$ cells were purified by FACS sorting from healthy donor human bone marrow (BM), whereas peripheral blood lymphocytes (PBL) purified from buffy-coat were used as differentiated cells. Since it was reported (Kosaki et al., 2000) that human Mest/Peg1 has an isoform-specific imprinting, two sets of oligos in RT-PCR were used to detect the expression of the two different isoforms, and as shown in Fig 30A both isoforms were expressed in stem/progenitor cells, although the alternative isoform seemed to be more specifically regulated. In order to assess Mest/Peg1 expression upon cell differentiation the following cell populations were used: $CD34^+$ $CD38^-$ (FACS-sorted from BM); $CD34^+$ $CD38^+$ (FACS-sorted from BM); $CD34^-$ (FACS-sorted from BM); PBL (from peripheral blood).

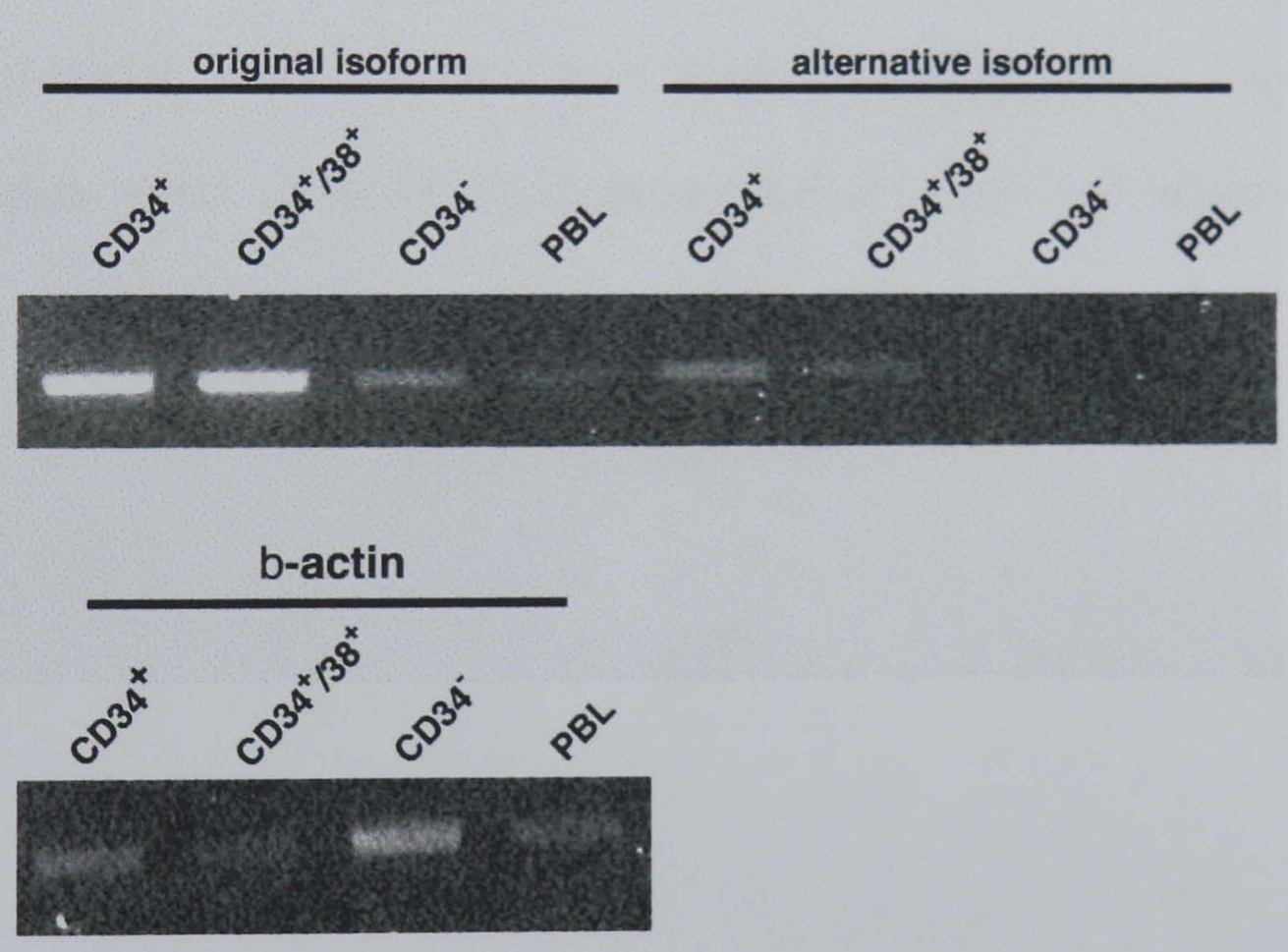
Interestingly, the Mest/Peg1 expression decreased as cells become phenotypical differentiated, with higher expression in $CD34^+$ $CD38^-$ and the lower in PBL (Fig. 30B). In sum, Mest/Peg1 was found specifically expressed in stem/progenitor cells of both neural and haematopoietic system, and its expression correlated with the degree of differentiation.

Fig. 30

A



B

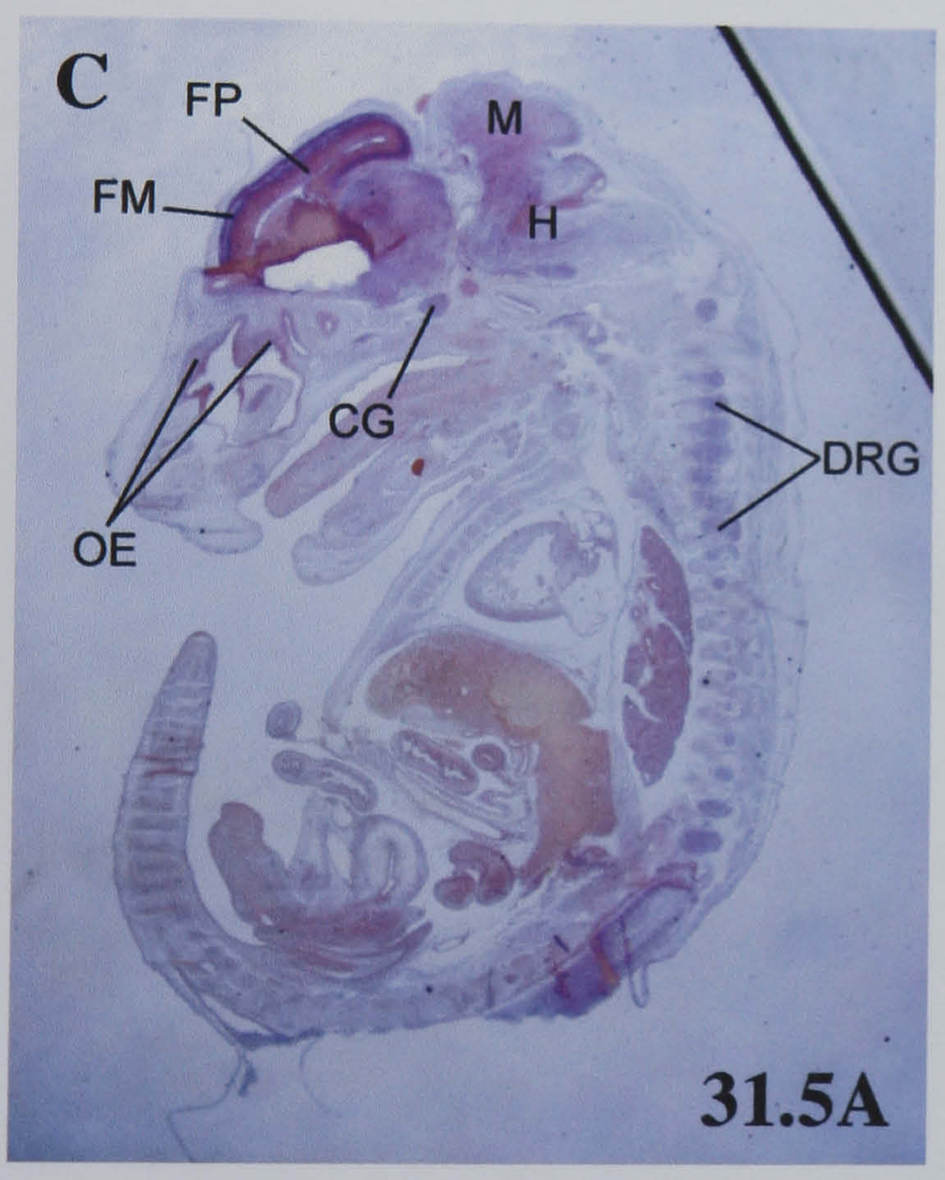


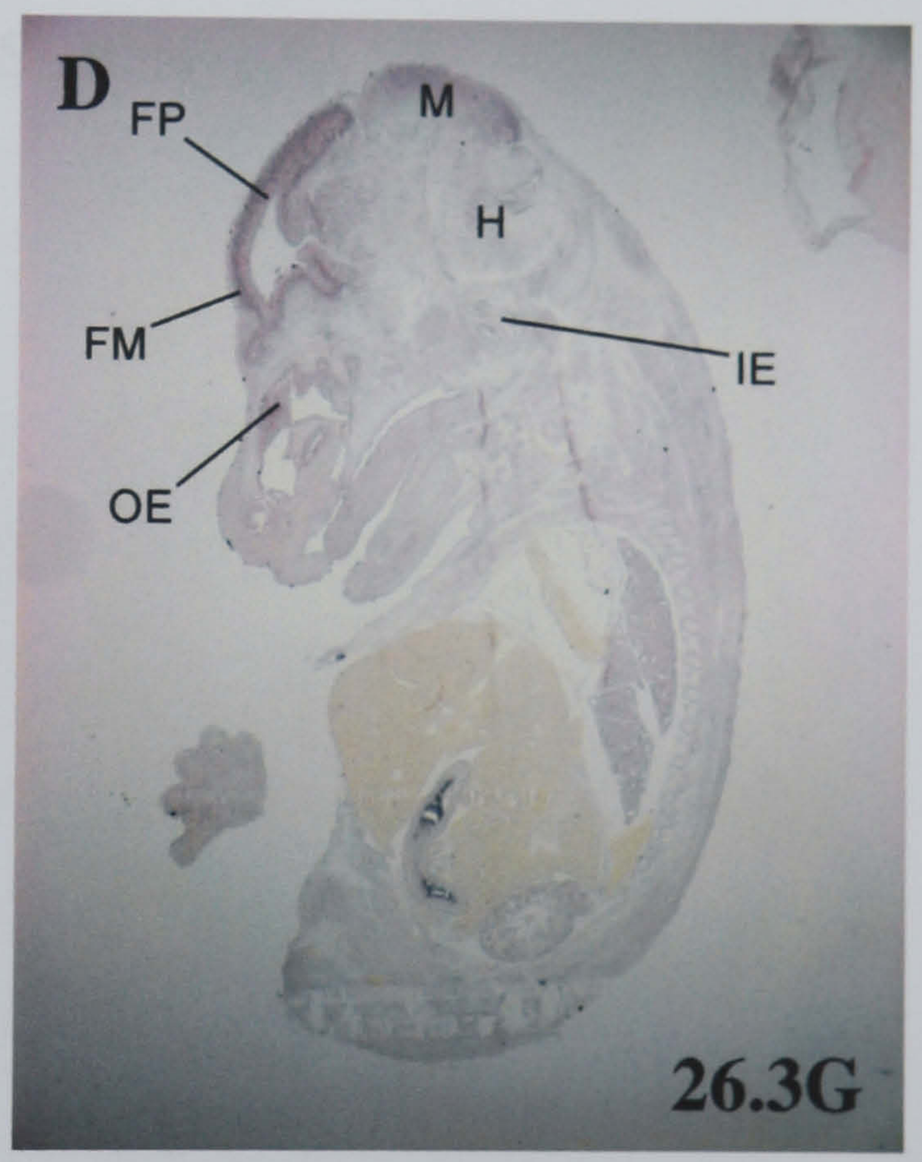
(A) Semi-quantitative RT-PCR analysis of Mest expression in human haematopoietic system. The original and alternative isoforms were checked in $CD34^+$ cells and peripheral blood lymphocytes (PBL). (B) Mest expression upon cell differentiation (from human bone marrow $CD34^+$ cells to peripheral blood lymphocytes)

Other differentially expressed *Tess* genes were analysed for their the expression patterns in development: *Tess* 29.5G (Hmgb2), *Tess* 21.5C (Sox4), *Tess* 31.5A (Prolactin M), and *Tess* 26.3G (Reticulocalbin). *Tess* clone 29.5G was found more expressed in stem cells (Fig.31A), *Tess* clone 21.5C and 31.5A in the progenitor population (Fig.31B, Fig.31C), and *Tess* clone 26.3G in differentiated cells (Fig.31D).

The *in situ* hybridization for clone 29.5G (Hmgb2) (Fig.31A) showed that this gene is specifically expressed in the actively proliferating ventricular zone of the dorsal developing forebrain. The expression of 21.5C (Sox4) (Fig.31B) and 31.5A (Prolactin M) (Fig.31C) instead was found low in the ventricular zone and high in the cortical plate. The in situ data obtained for these latter genes supported the microarray results, suggesting their possible role in the progenitor cells that leave the proliferative zone, migrate, and eventually differentiate in the proper layer. Finally, 26.3G (Fig.31D) (Reticulocalbin) was found expressed mainly in the cortical plate of the developing forebrain, confirming again the microarray data, which classified this clone as a specific marker of differentiated cells.

Fig. 31





RNA *In situ* hybridizations on WT mouse embryos of the Tes cDNAs differentially expressed in neural stem cells. The images correspond to sagittal sections of E14.5 mouse embryos hybridized to digoxigenin-labeled RNA probes. See results for description.

DISCUSSION

cDNA chip and library characterization

A cDNA chip specific for the developing telencephalon

One of the main aims described in this thesis was to develop a specific cDNA microarray containing about one thousand genes that are exclusively, or preferentially, expressed in the developing telencephalon. The spotted transcripts belong to a subtractive cDNA library (Porteus et al., 1992), which was obtained through a subtractive hybridization strategy between E14.5 and adult telencephalon cDNA libraries. This subtraction was performed in order to isolate genes that were involved in the various neurodevelopmental processes taking place in the E14.5 mouse telencephalon.

The task of realizing a "home-made chip" must be clarified, since the presence of a number of commercial microarrays could render this effort unnecessary. For mammalian species, most commercial DNA chips are designed on the basis of known genes or well characterized ESTs, and do not contain most of the rare or very tissue specific transcripts. Thus many developmental and neural-specific genes are likely to be missing in the so-called "genome-wide expression arrays". In fact, public databases are biased toward the most abundant expressed genes and ESTs, and because of the complexity of CNS development, neural specific genes are either less abundant or only expressed at specific moments or cell types. The sequencing and the bioinformatical analysis of the transcripts contained in the *Tess* library have demonstrated that this is the case. Of the one thousand different transcripts present on the *Tess* cDNA chip, 160 are completely missing in public ESTs database, and not all the *Tess* clones correspond to known ESTs (about five hundred) are present in the commercially available microarrays. Thus, the *Tess* array represents a valuable tool to investigate the expression profile of novel genes in the developing telencephalon. Furthermore using the array with such a specific collection of

developmentally regulated genes allows a more restricted dissection of the complicate molecular processes that take place during central nervous system development.

Subtractive cDNA library characterization: E14.5 telencephalic genes

The *Tess* gene expression profiling experiments comparing embryonic telencephalic cDNA versus adult cDNA confirmed the specificity of the subtractive library used to generate the microarrays. In fact, the majority of the *Tess* transcripts were more abundant in the E14.5 telencephalic RNA. The *in situ* hybridizations on E14.5 mouse sections allowed us to describe the expression patterns of both highly and moderately up-regulated developmental genes. As shown in Fig.11 and 12, for most of these transcripts the expression was restricted to the developing CNS, and for some of them the signal was specifically detected in the telencephalon (Fig. 11B, 11F, 12B, 12D, 12E). For some of these *Tess* cDNA clones the expression pattern was even restricted to particular areas of the telencephalon, demonstrating that the main goal of the project was achieved: to identify novel genes whose expression is confined to morphologically and topologically specific areas, since different regions of the telencephalon give rise to different structures and different cell compartments (for example generation of different classes of neurons or modes of migration). For instance, clone 21.7D (Fig.11B) is mainly expressed in the LGE of the basal telencephalon, or clone 12.6B is specifically expressed in the olfactory bulb, and to a lesser extent in the basal telencephalon.

Also the *Tess* cDNAs that were not found highly up regulated at E14.5 turned out to be very interesting, for example clone 19.5H (fold=2.53) showed a restricted expression pattern in the progenitor zone of dorsal telencephalon (Fig12D).

These results clearly prove that through the combination of microarray, and *in situ* hybridization expression profiling, it was possible to identify gene candidates having a potentially important role in telencephalic development. The combination of a high-throughput / low resolution (gene chip analysis) with low-throughput / high resolution (in

situ hybridization) methods has also allowed further selection of the *Tess* genes to be subjected to a comprehensive bioinformatics analysis. In the databases there is a large amount of sequence data (both genomic and EST sequences) that is extremely useful for the identification and structural characterization of novel genes, but to these many novel genes are still associated very few known functions. For instance, the databases are filled with "hypothetical proteins" or ESTs with unknown functions. Functional genomics strategies are supposed to eventually fill this gap, and the approach described in this thesis is in line with this attempt, and even if the novel genes identified in this work are still functionally "uncharacterised", nevertheless they can be categorized as developmental genes and, because of their restricted expression in the telencephalon, their potential role can be envisaged, and thus tested.

As an example, the sequencing analysis of the *Tess* clones mentioned above showed that six of them (out of twelve) correspond to novel genes: five are part of UniGene clusters of ESTs with unknown function, whereas one (clone 21.7D) has no corresponding sequence in the EST database. However, interestingly this *Tess* clone mapped in the first intron of the ribokinase (NCBI: BC023339) gene, an enzyme responsible for the phosphorylation of ribose. The presence of a transcribed sequence in an intron (in the same strand) may indicate the presence of a possible alternative splicing. Tissue-specific alternative splicing profoundly affects animal physiology, development and disease (Grabowski and Black, 2001), and genomic studies have suggested that 40 to 60% of all human genes are alternatively spliced (Mironov et al., 1999). The subtractive library used in this study turned out to be particularly rich in sequences corresponding to potentially alternative exons, since numerous clones map in intronic sequences. This additionally proves the importance of the *Tess* collection of genes used to realize the cDNA chip.

The *Tess* cDNAs corresponding to already known genes present in the array are also significant, for at least two reasons. First, they can be considered as markers useful for

validating the experimental results. For instance, Sox4 (clone 21.5C) is a transcription factor expressed at various stages of embryonic telencephalic development, and CD24a (clone 19.4G) is a glycosylphosphatidylinositol-anchored molecule that is expressed in migrating neurons of the developing mouse nervous system and in a small area of the adult SVZ. Secondly, there are known genes that were not known to be involved in telencephalic development, but that now have to be considered implicated in such process. For instance, clones 29.5G and 12.6B corresponded to Hmgb2 and Mest/Peg1, respectively. Interestingly, for both of them, because they were found expressed in the embryonic brain and in undifferentiated adult neural stem cells (see neural stem cells result), a role in development needs to be addressed (Fig.11E and 11F). Hmgb2 is known to be involved in chromatin modification, and chromatin remodelling may play a crucial role in stem cell biology and differentiation by regulating the intrinsic state of responsiveness of a cell (Doetsch, 2003). Mest/Peg1 may instead have a role in the transformation of toxic compounds, thus controlling cell homeostasis. Finally, the presence in the array of Cdo (clone 20.2D) and Crb1 (clone 11.3E) further proves that the microarray is a developmental specific gene collection, since both genes have been extensively characterized for having a role in development. Cdo expression was found to be spatially and temporally restricted during embryogenesis (Mulieri et al., 2000). Crb1, a mouse homologue of the *Drosophila crumbs*, is exclusively expressed in the embryonic eye and CNS, whereas in the adult its expression is confined to the regions of active neurogenesis (den Hollander et al., 2002).

Conclusions.

The aim of the first part of this thesis work was to develop a specific cDNA microarray for investigating the molecular aspects of telencephalic development. The design of this *Tess* cDNA chip was carefully studied, as the following precautions have been taken:

- 4 replicates for each spot (in order to perform statistical analysis).
- 40 known developmental gene markers (in order to have controls for the hybridization experiments),
- 10 artificial genes corresponding to yeast intergenic regions (in order to calibrate the dyes),
- negative control spots in many positions onto the array (to set the basal level of signal intensity).

The buffer composition and the conditions used to spot the clones have been selected in order to reach a high signal to noise ratio, thus allowing an accurate analysis of the data. Since the starting material (neural tissue or neural stem cells) was not abundant (2-3 cortices or subpallia of E14.5 mouse embryos, or a few million cultured neural stem cells), the protocol of labelling and hybridizations have been thoroughly designed, in order to obtain a high signal from 1 to 10 μg of total RNA.

This specific cDNA telencephalic chip has been used to compare E14.5 and adult telencephalic gene expression, and besides the evidence of the *Tess* subtractive library specificity, this comparison has allowed the identification of *Tess* clones exclusively expressed in the embryonic telencephalon.

Analysis of mouse models of telencephalic development

A number of transcription factors are part of the molecular network involved in the development of embryonic telencephalon. In the present work are described the expression profiles, obtained using the *Tess* array, of four different mutant mice null for telencephalic developmental genes. Two of these mouse models, Pax6 -/- and Ngn1/Ngn2 -/-, have been analysed for their molecular phenotype in the dorsal telencephalon. On the contrary, the Nkx2.1 -/- and Dlx1/Dlx2 -/- mutants show specific phenotypes, since Dlx1/Dlx2 are expressed in the ventricular and subventricular zones of the LGE and MGE, and Nkx2.1 is

exclusively expressed in the MGE ventricular zone and mantle layer. The analysis of these four mutants has allowed the identification of *Tess* cDNAs involved in the regulation of pallial and sub-pallial development. The results obtained in each mutant will be discussed, as well as an over-all expression profile of the mutants.

Tess array analysis of Nkx2.1 null mutant mice

The profiling of the Nkx2.1 mutant has provided few clones using the *Tess* array. The expression of this gene is restricted to the medial ganglionic eminence (MGE), and its mutation causes the MGE to be respecified to the more dorsal LGE. Since the tissue from which the RNA was extracted was the entire sub-pallium (MGE + LGE), it is possible to hypothesize that the transcripts regulated by Nkx2.1 have been diluted by the unaffected tissue. This dilution may be the cause of the mild differences observed in the study, especially for the rare transcripts. The only *Tess* clone that showed a significant up-regulation was 12.6B, corresponding to Mest/Peg1 gene, which is significantly expressed in the proliferative regions of the basal telencephalon and olfactory bulb. Since Nkx2.1 is expressed in the proliferative and postmitotic cells of the MGE, and its mutation causes a misspecification of the MGE into the more dorsal LGE, the over-expression of the Mest gene, normally confined to the proliferative stem cell population (see its up regulation in neural stem cells), may mean that MGE cells retain the expression of Mest/Peg1 and do not differentiate. In fact, in the Nkx2.1 mutant there is a lack of cells migrating to the cortex (cortical interneurons) and to the striatum (cholinergic neurons).

Tess array analysis of the Dlx1 and Dlx2 double mutant mice

The gene expression profiling of the Dlx1/Dlx2 mutant mice has identified a number of down-regulated *Tess* transcripts, but only the ones with at least a four-fold difference have been further analysed. Despite the excellent R^2 value (Fig.16), reflecting the well-designed

and performed microarray hybridization, only the "four fold down-regulated genes" were first analysed, this for at least two reasons:

(i) the marker Gad65 was found down-regulated about five times, and this gene was previously described as a direct target of the DLX proteins (Stuhmer et al., 2002a), thus the decision was made to analyse transcripts above and around to this threshold;

(ii) the process of array data normalization is based on the assumption (formulated on the basis of large dataset of sequences, for example 15.000 clones) that the overall level of expression is identical in the two samples, but working with small and tissue specific arrays (as in our case) implies that most transcripts are differentially represented in the two samples. Therefore, the normalization process, forcing the data to reach an overall equal balance of expression, could remove a number of differentially expressed genes, and in order to avoid this loss, our data was not normalized but simply balanced with the intensities of the yeast DNA intergenic regions present in the array. As a result, the threshold of four fold times had to be applied on the gene expression differences, as a stringent strategy to limit false positive data.

The data obtained through the analysis described above was then confirmed and validated by performing RT-PCR and *in situ* hybridizations on WT and KO tissues.

The sequence analysis of the differentially expressed *Tess* genes revealed that some were completely novel, some corresponded to uncharacterised ESTs, and some to known genes. Among the down-regulated known genes clone 8.8G, corresponding to Bcl11a, revealed an interesting pattern of expression in the sub-pallium by *in situ* hybridization. Bcl11a (also called Evi9) is a Kruppel zinc finger gene functioning as a myeloid or B cell proto-oncogene in mice and humans, respectively, and is essential for postnatal development and normal lymphopoiesis. Bcl11a mutant mice completely lack B cells, and have alterations in

the T cells compartment. Phenotypic and expression studies demonstrated also that Bcl11a functions upstream of the transcription factors Ebf1, and Pax5 in B cells (Liu et al., 2003). This gene has never been reported to be expressed in the nervous system, while the downstream Ebf1/Olf-1 gene, which belongs to a small multigene family encoding closely related helix-loop-helix transcription factors, plays an important role in neuronal differentiation. More specifically, Ebf1 controls cell differentiation in the murine embryonic striatum, and its mutation affects the postmitotic cells leaving the subventricular zone (SVZ) on their route to the mantle (Garel et al., 1999), and the mantle of the striatum is one of the main regions where clone 8.8G/Bcl11a is expressed (Fig.20). On the basis of these results, a genetic hierarchy underlying the specification of striatal neurons of the basal telencephalon can begin to be envisioned, where Dlx1/Dlx2 operate (directly or indirectly) upstream of Bcl11a, which in turn operates upstream of Ebf1. Partial evidence for this hypothesis comes from the observation that in Ebf1 mutant mice the expression pattern of Dlx1 is unaffected (Garel et al., 1999), while Dlx5 is expressed also in the mantle suggesting that Dlx1/Dlx2 (expressed in VZ/SVZ) are required for expressing Dlx5 in the SVZ while Bcl11a/Ebf1 for repressing its that expression in the mantle (Garel et al., 1999).

Tess expression profiling of Dlx1/2, Pax6 and Ngn1/2 null mutant mice: a molecular dissection of telencephalic development.

The gene expression profile studies on wt and mutant mice for these different transcription factors have provided important data for the dissection of the genetic network controlling telencephalic development. As shown in Table 13, by clustering the results of the three different expression profiles the differentially expressed *Tess* genes could be classified into two classes:

(i) the *Tess* clones both down-regulated in the Dlx mutant basal ganglia primordium and up-regulated in the Pax6/Ngns mutant cortex (so called "basal ganglia specific genes");

(ii) the *Tess* clones specifically down-regulated in both pallial Pax6 -/- and Ngn1/2 -/- mutants (so called "cortex specific genes")

Table 13

Tess transcripts disregulated in telencephalic mutants analysis

A

basal ganglia specific genes

clone	*Description*	*Dlx1/Dlx2 -/-*	*Ngn1/Ngn2 -/-*	*Pax6 -/-*
28.8E	Novel	-46,51	+9,02	+4,34
31.5E	Novel	-6,44	+5,04	+3,15
19.3E	Foxp2	-4,11	+1,02	+1,76
23.10E	Novel	-4,5	-1,3	+1,98
{Gad65}		-5,22	+4,36	-

B

cerebral cortex specific genes

clone	*Description*	*Pax6 -/-*	*Ngn1/Ngn2 -/-*
9.10F	Novel	-2,39	+1,08
13.12G	Novel	-2,3	-2,03
{Tbr1}		-2,1	-1,7
6.7H	Novel	-2,03	-2,07
18.6C	Novel	-1,92	-1,17
{Tbr2}		-1,92	-2,52
18.7E	Novel	-1,40	-3,82
26.9E	Trim 32	-1,27	-2,41

Schematic representation of *Tess* transcripts identified through the microarray analysis of the mutant mice: (A) table showing the list of genes down-regulated in Dlx1/Dlx2 and up-regulated in Pax6 and Ngn1/Ngn2 mutant mice, (B) table showing the list of genes down-regulated either in Pax6 mutants and/or in Ngn1/Ngn2 mutants. In brackets are reported the known genes included as markers, in the *Tess* gene chip. The clones that are down regulated in both mutants are underlined.

Two *Tess* cDNAs, 28.8E and 31.5E, were found significantly down regulated in the ganglionic eminence of Dlx1/Dlx2 mutant mice, and also up-regulated in the pallium of Ngn1/Ngn2 and Pax6 mutant mice (Table 13A). This finding has to be related to the pallial (cortical) dorsal to ventral transformation in both Pax6 and Ngn1/Ngn2 mutant mice. In the Pax6 mutant it has been shown that there is a severe defect of the dorso-ventral patterning (Stoykova et al., 2000), in particular there is an early developmental ventralization of the neuroepithelium at the pallial/subpallial border, and for this reason the morphogenesis of the basolateral cortex appears to be strongly affected. The pallial/subpallial border is a region where the *Tess* clone 28.8E is naturally expressed (Fig.20 and Fig. 22D-K), and so the expansion of its expression in the mutant cortex is in agreement with the molecular ventralization of the of Pax6 -/- pallium. In the Ngn1/Ngn2 double KO a similar phenomenon has been observed: neurons born in the pallium fail to completely differentiate into the characteristic cortical pyramidal projection neurons and start instead to express ventral telencephalic markers (like Gad65, a marker gene included in this study: Table 4A).

Regarding the other *Tess* clones reported in Table 13A, *Tess* clones 19.3E and 23.10E are also significantly up-regulated in the Pax6 mutants. Clone 19.3E corresponds to Foxp2, a member of the Fox family of winged-helix/forkhead transcription factor genes, and while clones 28.8E and 31.5E are expressed in the VZ/SVZ of the subpallium, the Foxp2 transcript is mainly detected in the post-mitotic layers of both cerebral cortex and striatal anlage (Ferland et al., 2003). These different distributions of the 28.8E/31.5E and 19.3E cDNAs within the cortical wall suggests their involvement in different processes: proliferation or specification for clones 28.8E and 31.5E, and differentiation or migration for clone 19.3E

The expression profiling of the *Tess* genes in the Ngn1/Ngn2 double KO mice has provided new clues about the molecular profile controlled by these two bHLH transcription

factors (Table 13B), since several disregulated *Tess* transcripts correspond to novel genes, and others map into introns of known genes indicating again the extensive alternative splicing occurring in brain development. The finding of Gad65 up regulation in this context serves as validation of the cDNA chip analysis, since this gene was previously identified by *in situ* hybridization to be ectopically expressed in the cerebral cortex of Ngn1/Ngn2 double mutants (Fode et al., 2000). The same marker was not found up regulated in the Pax6 mutants, indicating that the dorsal to ventral transformation that occurs in the two mutant models is molecularly different. This data is in agreement with the fact that Mash1 expression (a marker for relatively undifferentiated neuroblasts) has been shown to be unchanged in the ventricular /subventricular zone of the Pax6 mutant brain (Stoykova et al., 1996), while in Ngn1/Ngn2 mutant pallium Mash1 is strongly up-regulated (Fode et al., 2000). Also the *Tess* clones 28.8E and 31.5E were differentially up-regulated in Ngn1/Ngn2 mutants compared to the Pax6 mutants (Table 13A), probably because the over-expression of Mash1, an early important regulator of neurogenesis in the ventral telencephalon, may induce a striking change in regional respecification, leading to the higher expression of ventral telencephalic markers in the Ngn mutants.

Also the expression profiling of the Pax6 mutant pallium has generated new data on the molecular network involved in cortical development. The analysis of the pallium, a particular region of the developing CNS, should in fact facilitate the identification of specific Pax6 target genes in this tissue. Among the up-regulated genes, the presence of Foxp2 is of interest (*Tess* clone 19.3E), a gene expressed both in basal and dorsal telencephalon, and of seven *Tess* clones corresponding to novel genes.

Four cDNAs (13.12G, 6.7H, Tbr1, Tbr2) were down regulated in both mutants (Table 13B), while others were down regulated only in one of the two mutants, suggesting that the genetic program under the control of these transcription factors is partially different, and since Pax6 has been demonstrated to be upstream of Ngn2 (Scardigli et al., 2001), the

presence of Ngn1/Ngn2 specific down regulated genes probably implies an additional important contribution of Ngn1 in setting up a proper molecular machinery for cortical development.

Tess clone 31.5E and telencephalic development

Tess 31.5E was one of the transcripts showing a complementary expression profile in the three mutant forebrains: it was down regulated in Dlx1/Dlx2 mutant subpallium and up regulated in Ngn1/Ngn2 and Pax6 mutant cortex. As described in the results section, there is evidence that this cDNA corresponds to an antisense transcript: sequence bioinformatic analysis using AntiHunter algorithm (http://bio.ifom-firc.it/ANTIHUNTER/), its identification in the Dlx5/Dlx6 intergenic region, and the recent discovery of the extensive role of antisense sequences in development.

In *Drosophila* recent studies demonstrate the importance of intergenic regulatory sequences in the modulation of expression of developmental genes, in particular intergenic transcription is required early in embryogenesis to initiate the activation of the *Drosophila* bithorax complex and to define the domains of activity for the infraabdominal region (*iab*) cis-regulatory elements (Bae et al., 2002; Drewell et al., 2002).

It has been hypothesized that their transcription is necessary and sufficient to "open" the chromatin, recruit specific factors that bind regulatory regions, and initiate the transcription of the specific set of genes. This seems to be supported by the nuclear localization of these transcripts. This localization may indicate that they have no cellular function except their transcription (Bae et al., 2002; Drewell et al., 2002).

Two studies have been published on the presence of highly conserved sequences in the intergenic regions of the Dlx1/2 and Dlx5/6 genes: (i) I56i and I56ii sequences in the Dlx5/Dlx6 locus; (ii) I12a and I12b sequences in the Dlx1/Dlx2 locus (Ghanem et al., 2003; Zerucha et al., 2000). These sequences extend over a few hundred base pairs, and are the potential binding site of a large number of regulatory factors. Furthermore, it has been

demonstrated that mouse I56i sequence can efficiently target reporter gene expression to the forebrain in 100% of the transgenic mice, and that the efficiency of I56i was higher than I56ii (Zerucha et al., 2000).

The sequence analysis of clone 31.5E showed that it is part of the two already known sequences, two Riken transcripts (AK032537 and AK038694) which contain part of the I56i sequence, and we have proved by RT-PCR that these sequences are transcribed in WT subpallium, and down regulated in Dlx1/Dlx2 mutants (Fig.19).

Conclusions

As already mentioned, most of the commercially available cDNA chips are developed using public ESTs databases as sources of transcripts to be spotted on the arrays. As a consequence, rare transcripts, specifically expressed in restricted domains of the developing telencephalon, are often missing in these arrays. On the contrary the *Tess* array of rare, embryonic and specific transcripts, has allowed the identification of novel genes specifically expressed in the developing telencephalon and directly, or indirectly, regulated by the transcription factors: Dlx1-2, Pax6, Nkx2.1, and Ngn1-2.

In summary, the *Tess* microarray analysis has identified 69 different *Tess* transcripts differentially regulated in the double Dlx1-2, Pax6, Nkx2.1 and double Ngn1-2 null mutant mice, and 44 of these didn't correspond to any known gene. Moreover, for 12 of the 44 novel genes, only the corresponding genomic sequence was present in the database. Among the known genes, some have not been known to be involved in telencephalic development: for example the Mest/Peg1 gene (up regulated in the Nkx2.1 -/-; Mest/Peg1 has also been found as preferentially expressed in undifferentiated neural stem cells), and the Bcl11a gene (down regulated in the Dlx1-2 -/-).

The profiling of these mouse models and the computational analysis of the data generated by the 4 microarray experiments, has identified at least two novel genes (*Tess* clone 31.5E

and 28.8E) candidates for playing a major role in telencephalic development. In particular, these *Tess* are specifically expressed in the embryonic subpallium, which gives rise to important structures of the adult brain (the striatum and the pallidum, for example), and from which GABAergic interneuron precursors originate and migrate to reach the cerebral cortex.

Tess clones 31.5E and 28.8E have been analyzed by RNA *in situ* hybridizations both in WT and in Dlx1-2 null mutant telencephalon, and the down-regulation found by microarray analysis has been confirmed. Interestingly, the different levels of down-regulation (in Dlx1-2 -/- subpallium) reported by microarray hybridization for the two *Tess* clones (46,51 for 28.8E and 6,44 for 31.5E) have been also confirmed by RNA *in situ* hybridization. In fact, the signal for *Tess* 28.8E was completely absent in the Dlx1-2 null mutant brain, whereas the signal for 31.5E could be still detected in specific structures of the mutant subpallium (the septum, for example).

These two candidates clones are also interesting for their sequences. As discussed above, for *Tess* clone 31.5E there are evidence for it being a non-coding RNA involved (together with Dlx1 and 2) in the transcriptional regulation of the Dlx5 and 6 genes. By looking in the Riken database (http://genome.gsc.riken.go.jp/) it is possible to note that half of these transcripts can be considered non-coding RNAs (Okazaki et al., 2002), and recently also microRNAs (a class of smaller non coding RNA) have been extensively studied for their involvement in gene expression regulation (Chen et al., 2004). Thus probably in the near future the study of non-coding RNAs will represent a major focus in the field of molecular developmental biology.

Even if at present there are no definitive data about the full-length sequence of *Tess* clone 28.8E, its position in a genomic region characterized by the presence of few ESTs or predicted transcripts indicates that this transcript belongs to a completely novel gene. Its expression in specific proliferative regions of the basal telencephalon suggests its

involvement in the generation of the subpallial neurons, fated either to become striatal/pallidal neurons or to migrate dorsally as interneurons. In conclusion, this part of the project allowed the identification of two novel genes, candidates for being regulated by the Dlx transcription factors and for being involved in the development of the basal telencephalon.

Adult Neural Stem Cells

The rationale behind the expression profiling experiments on adult neural stem cells was that the restricted and specific collection of *Tess* genes could be useful to unravel some of the molecular mechanism underling neural stem cells proliferation and differentiation. Few papers have been already published on the microarray analysis of CNS stem/progenitors cells (Geschwind et al., 2001; Karsten et al., 2003; Luo et al., 2002). There are, however, substantial differences in the approach I have undertaken:

i) the use of a pure population of neural stem cells (the so called "doublets") in comparison with neural progenitor and differentiated cells (in a similar approach Terskikh *et al.*, 2003, used haematopoietic stem cells and a series of progenitors populations)

ii) the use of the *Tess* array, containing mostly unknown, or uncharacterised genes specifically involved in developmental processes such as neural proliferation and differentiation.

Furthermore, between the two main approaches of assessing the profile and clustering of a large set of transcripts, and the identification of a restricted number of genes which can be then studied in a more detail, it has been decided to select a small subset of developmental genes that are differentially expressed during the process of neural differentiation. In particular, in this study 22 different *Tess* cDNAs were identified and analysed. They have been characterized for their sequence homology, validated by RT-PCR, and examined for their tissue expression by RNA *in situ* hybridization.

The cluster analysis of the differentially expressed *Tess* genes in the three populations of neural cells (Table 12 and Fig.27) has been able to group genes with the same pattern of expression upon differentiation:

1. *Tess* genes specifically expressed in the neural stem/progenitor cells;

2. *Tess* genes specifically expressed in differentiated cells;

3. *Tess* genes equally expressed in progenitor and differentiated cells.

Stem/progenitor cells genes.

Table 14 shows the subset of genes that, because of their preferential expression, can be defined as "stem specific genes".

Table 14

Clone	Nsc	Prog	Diff
12.6B (Mest/Peg1)	1	1	-6,53
18.11A (Foxg1/Bf1)	1	1	-5,4
23.3D (Novel)	1	-1,9	-4,76
29.5G (Hmgb2)	1	-1,81	-3,7
13.6H (Sox11)	1	1	-3,31
21.12E (Cyclin D2)	1	-2,4	-3,03
18.9A (Rad23b)	*1*	*1,42*	*-2,98*
9.5A (Novel)	*1*	*2,1*	*-2,91*
27.6A (Novel)	1	1	-2,7
13.6C (Stag2)	1	1	-2,46
7.3G (Pcmt1)	1	-1,91	-1,72

(Nsc: undifferentiated neural stem cells; Prog: neural progenitor cells; Diff: differentiated cells).

They are in fact all down regulated in the differentiated cells, while in the progenitor and stem cells these genes are expressed at a comparable level, but with the exception of clones 9.5A and 18.9A (in italic in Table 14) which seem to be more up-regulated, and thus more specific, for progenitors. Based on the sequence analysis, it is possible to organize these genes into several functional classes (Table 15), all highly relevant for stem cell biology:

Table 15

Category	Total	Clone
Cell Cycle	2	21.12E: 13.6C
Transcription	2	18.11A; 13.6H
Chromatin	1	29.5G
DNA repair	1	18.9A
Detoxification	2	12.6B: 7.3G
Novel	3	27.6A: 23.3D, 9.5A

Cell cycle genes.

Clone 21.12E corresponds to Cyclin D2. The cyclins are a well-characterized family of kinases that have a crucial role in cell cycle. In particular the cell cycle activation is coordinated by the D-type cyclins, which are rate limiting and essential for the progression through the G1 phase of the cycle. Cyclin D2 plays an essential role in the response to mitogens, and in transmitting the signal to the RB and EF2 pathway. It has been shown that Cyclin D2 expression is highly restricted to the brain (Ross and Risken, 1994), and in particular only to precursor cell populations, for example the proliferating granule neuroblasts of the cerebellum. In situ hybridizations and western blot analyses demonstrated also that its expression is dramatically reduced in adult brain (Tamaru et al., 1994).

Clone 13.6C corresponds to Stag2, which belongs to the stromalin (SA) family of nuclear proteins (Carramolino et al., 1997). SA proteins are specific cohesin complex subunits that maintain sister chromatid cohesion in mitosis and meiosis, and in *Drosophila* (Valdeolmillos et al., 1998) are expressed mainly in neurogenic territories during embryonic development. A recent paper (Prieto et al., 2002) has demonstrated that Stag2 and Rad21 form a cohesin complex in meiosis.

Transcription factors.

Clones 18.11A and 13.6H both correspond to well known transcription factors: Foxg1/Bf1 and Sox11 respectively. Both genes are critical for the development of the central nervous system. The winged helix gene *Brain factor-1* (BF1) has a pleiotropic role in the

development of the cerebral hemispheres of the brain. Mice lacking BF1 have defects in the morphogenesis of dorsal and ventral telencephalic structures. Interestingly, it has been shown that telencephalic progenitor cells lacking BF1 differentiate into neurons prematurely, and there is an early lengthening of their cell cycle (Hanashima et al., 2002). Hanashima et al. also demonstrated that BF1, while promoting the proliferation of telencephalic progenitor cells (thorough a DNA binding-independent mechanism), delays neuronal differentiation in the neocortex (through a DNA binding-dependent mechanism). These data indirectly validate our findings, and corroborate the hypothesis that our collection of developmental genes preferentially expressed in the telencephalon at E14.5 represents a useful tool for investigating and restricting the molecular profiling of adult stem cells.

Another known transcription factor that plays a relevant role in embryonic development, and in the control of NSC proliferation, is Emx2 (Galli et al., 2002), and it is reasonable to hypothesize, based on our data, a similar role also for BF1.

Sox11 is a member of the *Sox* family of developmentally regulated transcription factors, which is characterized by the presence of a highly conserved HMG-domain responsible for a sequence specific DNA binding. The *Sox* genes are categorized in seven groups according to their sequence similarity. Sox11 falls in the same group as Sox4 (group C), since they exhibit a high sequence homology both within, and outside the HMG-domain (Sox4 was also detected in our analysis, and it will be discussed below). Sox11 expression data has suggested a role in neuronal maturation (Hargrave et al., 1997), since it is mainly expressed in the sub-ventricular zone, where neural precursor cells generally exit mitosis and begin to differentiate. Our finding of a preferential expression of Sox genes (Sox11 and Sox4) in adult neural stem cells is consistent with some recent studies which also demonstrated a role of the homologous Sox2 (Graham et al., 2003) and Sox10 (Kim et al., 2003) in neural progenitor cells.

Chromatin binding.

Tess clone 29.5G corresponds to the Hmgb2 gene, a member of the Hmgb gene family (Hmgb1, -2 and -3). HMBG1, the best known member of the family, acts as an architectural protein which bends DNA, and promotes protein assembly on specific DNA targets. Hmgb2 is highly homologous to Hmgb1 (more than 80% amino acid identity), and it interacts with chromosomes in a highly dynamic manner in mitotic cells (Pallier et al., 2003), proving the involvement of this family in chromatin binding and remodelling. Chromatin is remodelled during cell cycle progression and in multipotent haematopoietic cells it has been proved that the lineage-associated genes have their regulatory genomic regions accessible before the final cell commitment (Hu et al., 1997). Chromatin remodelling leads to various degrees of DNA accessibility to transcription factors, which in turn regulate the patterns and levels of gene expression. These data together with the finding that Hmgb2 mutant mice have reduced fertility due to a specific impairment of germ cell differentiation (Ronfani et al., 2001) indicate that Hmgb2 is a potential major player in the biology of the neural stem cells. These cells are multipotent, and thus retain the capacity to initiate the expression of lineage specific genes when exposed to differentiating signals. On the contrary, progenitor cells (and of course differentiated cells) are already committed, and have a more restricted repertoire of gene to express due to a remodelled and more compacted chromatin.

DNA repair.

Clone 18.9A corresponds to Rad23b, a gene involved in DNA repair after UV damage, and its expression in stem/progenitors cells is in line with its function since the high rate of proliferation requires a tight control during DNA replication, in order to avoid error transmission in daughter cells.

Detoxification genes.

The *Tess* clone 12.6B corresponds to Mest/Peg1, and clone 7.3G to Pcmt1. The high expression of the Mest/Peg1 gene in the NSC is a very intriguing finding, since it is an imprinted gene, and paternally expressed. Some genes are active and expressed only on one of the parental genome, as a result of genomic "imprinting", a process that confers functional differences on parental genomes during mammalian development. Mice mutant for the Mest/Peg1 gene (Lefebvre et al., 1998) have no evident phenotype except for an embryonic growth retardation associated with reduced postnatal survival rates, and abnormal maternal behaviour and impaired placentophagia. Regarding its putative function, the sequence similarity with a α/β hydrolase folding suggests an enzymatic role. Epoxide hydrolases, characterized by this specific folding, belong to a subgroup of hydrolytic enzymes that catalyze the hydration of chemically reactive epoxides. Epoxides (Fretland and Omiecinski, 2000) are organic "three-membered" oxygen compounds that arise from oxidative metabolism of endogenous, as well as xenobiotic compounds via chemical and enzymatic oxidation processes. The resultant epoxides are typically unstable in aqueous environments and chemically reactive and, for this reason, are supposed to be mutagenic and carcinogenic initiators. These enzymes have probably an important role in stem cell biology since detoxification means more probability to survive. Reduced stem cells survival may impair their regenerating ability, and result in a decrease production of committed progenitors, and eventually terminally differentiated cells.

Besides being up regulated in the NSCs, the expression level of Mest/Peg1 was verified also in stem/progenitors cells of the human haematopoietic system: RT-PCR analysis showed a striking decrease in expression as these cells differentiate (Fig. 29A and B). Moreover, *in situ* hybridizations demonstrated that during telencephalic development Mest/Peg1 is specifically expressed in proliferative VZ/SVZ regions where stem/progenitor cells reside (Fig. 29). In adult tissues this gene is mainly expressed in the brain (Lefebvre et al., 1998), but not in the neurogenetic areas (lateral wall of the lateral

ventricle, hippocampus, rostral migratory stream). Nevertheless it has to be considered that the up-regulation detected via microarray hybridization was obtained in adult neural stem cells cultured in the presence of FGF2 and EGF, conditions in which these cells proliferate at high rate. Thus Mest/Peg1 seems to be highly expressed when stem/progenitor cells need to proliferate a lot (such as in embryonic telencephalic development), but its expression decreases as these cells become quiescent, as in the adult. The finding that Mest/Peg1 is expressed in the human adult stem/progenitor cells of the haematopoietic system is in apparent contrast with the data on adult brain, but it has to be considered that in hematopoietic stem cells the transcript was detected by RT-PCR (a more sensible technique than *in situ* hybridization), and that the hematopoietic stem cell compartment is subjected to a high renewal rate.

Pcmt1 (L-Isoaspartyl O-methyltransferase) belongs to a similar class of enzymes, since it repairs damaged proteins (with accumulated abnormal aspartyl residues) during cell aging. This "repair" reaction helps to maintain the proper protein conformation by preventing the accumulation of damaged protein with abnormal amino acid residues. Interestingly mice mutant for Pcmt1 (Kim et al., 1999) suffer fatal seizures and retarded growth (this aspect is similar to Mest/Peg1 mutant mice).

Novel genes.

Tess clones 9.5A, 27.6A and 23.3D had no homology with known genes. Clone 23.3D belonged to the UniGene cluster Mm.45361, and by BlastX analysis it was possible to identify WD40 domains in its sequence. The WD-repeat proteins are found in all eukaryotes and are implicated in a variety of functions (Smith et al., 1999). The best-characterized WD-repeat protein is the Gβ subunit of the heterotrimeric G proteins, which forms a tight dimer (Gβγ) with the Gγ subunits. However, the proteins containing WD repeats are involved in a broad spectrum of important eukaryotic functions: RNA-processing complexes, transcriptional regulators, cytoskeleton assembly, mitotic spindle

formation, cell division, vesicle formation and vesicular trafficking. Since these proteins can be involved in different processes related to stem/progenitor cell biology, it is difficult to hypothesize a specific role for this novel gene. Clone 9.5A corresponded to a completely unknown gene since by Blast analysis it was only possible to match its sequence to the mouse genome (chromosome 16C13) in a region where no ESTs are reported. Also clone 27.6A didn't match any transcribed sequences, or ESTs, but interestingly it is localized in the sixth intron of the Calmodulin binding protein 3 gene (Striatin). This may be of interest, since in the *Tess* library there was at least another clone, 20.6D, that matches in the seventh intron of the same gene. The region where 27.6A maps is very complex, and comprises at least two other overlapping genes: Ap4s1 (adaptor-related protein complex AP-4 sigma 1) and Striatin. For this reason clone 27.6A may correspond to a new transcript, or to an alternative splicing of Ap4s1 or striatin.

Progenitor/differentiated cell genes

Table 16 shows the subset of genes selectively up regulated in the progenitor population and in the differentiated cells.

Table 16

clone	**Nsc**	**Prog**	**Diff**
21.5C (Sox4)	1	2,68	2,68
8.12C (Vrk1)	1	2,47	2,27
30.2D (Polybromo 1)	1	2,55	2,04
31.5A (Prolactin like M)	1	2,1	2
18.11E (Tubulin beta IV)	1	2,5	1,26

These genes can be organized into functional classes, as follows (Table 17):

Table 17

Category	**Total**	**Clone**
Kinase	1	8.12C
Transcription	1	21.5C
Chromatin	1	30.2D
Cytoskeleton	1	18.11E
Hormone	1	31.5A

Kinase.

Clone 8.12C corresponded to Vrk1 (vaccinia-related kinase 1). The vaccinia-related kinase proteins (VRK1, 2 and 3) are Ser-Thr kinases, and act as upstream regulators of several transcription factors. It has been demonstrated that human VRK1 phosphorylates murine p53 protein in a threonine residue (Lopez-Borges and Lazo, 2000), suggesting that VRK1 is a regulator of this protein. Recent work (Vega et al., 2003) has analysed the expression pattern of the three VRK proteins during murine haematopoietic development. The corresponding three genes are expressed in foetal liver and peripheral blood, with the highest levels between E11.5 and E13.5, a time when liver cells proliferate massively. Vrk genes are also expressed, particularly at mid-gestation, in the embryonic thymus and spleen, while in the adult they are undetectable. Therefore, these genes are directly involved in embryonic development (at least in the haematopoietic system), and thus their expression in adult neural stem cells may indicate a similar involvement in the control of proliferation and differentiation.

Transcription factor.

Clone 21.5C corresponded to Sox4, another member, together with Sox11 (see above) of the *Sox* gene family; Sox4 is expressed in a wide range of tissues, and has been shown to be functionally involved in heart, B-cell and reproductive system development. In general Sox2, Sox4, and Sox11 are all highly expressed in the developing neural tube. At early stages (up to E12) the strongest expression of Sox4 is detected in the SVZ, where neural precursors cells have left the mitotic cycle and start their differentiation (Cheung et al., 2000). Cheung et al. have suggested that Sox4 expression is up-regulated when neuronal precursor cells leave the proliferative ventricular zone of the neural tube and migrate radially, starting their differentiation into mature neurons (Cheung et al., 2000). Interestingly, at P0 Sox4 continues to be expressed in the entire cortical plate, suggesting a role in late cortical neuron differentiation and maturation.

These findings are in agreement with our experimental data on NSCs differentiation, since Sox4 was more expressed in the progenitor and differentiated cells.

Chromatin remodelling.

Clone 30.2D corresponded to Polybromo1. Its protein sequence is characterized by the presence of a particular domain: the bromodomain. Bromodomains are a family of protein modules originally found in proteins associated with chromatin and in nearly all nuclear histone acetyltransferases (Zeng and Zhou, 2002). They have been recently discovered to function also as acetyl-lysine binding domains, which are supposed to regulate protein-protein interactions in processes like chromatin remodelling and transcriptional activation. Transcriptional activation is a key process in cell differentiation, since cells have to activate all the genes required for lineage progression. The identification in both stem and progenitor cells of Hmgb2 (*Tess* clone 29.5G) and Polibromo1 (*Tess* clone 30.2D), which are both involved in chromatin remodelling, is significant since this aspect in stem cell biology is now a matter of intense investigation (Doetsch, 2003; Molofsky et al., 2003).

Cytoskeleton.

Tess clone 18.11E corresponded to Tubulin beta IV. Remarkably this clone in the microarray analysis is up regulated only in the progenitor population, while it is equally expressed at a lower level in the stem and differentiated cells. This finding could be due to the fact that the *in vitro* differentiation of the NSCs leads to the generation of a mixture of mature neural cells (neurons: 35%, oligodendrocytes: 10% and astrocytes: 55%), and the beta-tubulin is considered specific only for the neurons. Nevertheless, recently it has been demonstrated that Tubulin beta II is expressed in neural stem/progenitor cells and in radial glia during human foetal brain development (Nakamura et al., 2003), and this is consistent with our finding of Tubulin beta IV being highly expressed in progenitor cells.

Hormone.

Clone 31.5A corresponded to Prolactin like-protein M. This gene was identified in a recent study (Toft and Linzer, 2000), and it belongs to the prolactin/growth hormone family. These hormones have a spectrum of cell and temporal specific patterns of expression. In particular they are mainly involved in trophoblast differentiation, where they regulate programs of trophoblast-specific gene expression (Toft and Linzer, 2000).

It is difficult to hypothesize a role for this gene in progenitor and differentiated cells on the basis of these information. The presence of secreted factors may be related to the cell-cell communication during neural stem cell differentiation.

Differentiated cell genes.

Table 18 shows the subset of up-regulated genes in differentiated cells.

Table 18

clone	Nsc	Prog	diff
32.4D (Connexin 43)	1	6,09	14,43
22.10C (Zfp131)	1	-1,85	5,42
26.3G (Reticulocalbin)	1	2,22	4,59
9.4G (Slc16a4)	1	2	3,06
28.1B (Calponin 3)	1	1	3,03
30.12E (Novel)	1	1	3

These genes belong to the following functional classes, (Table 19):

Table 19

Category	Total	Clone
Novel	1	30.12E
DNA binding	1	22.10C
Transporter	1	9.4G
Ca^{+} binding	2	26.3G; 28.1B
Cell-Cell communication	1	32.4D

Novel genes.

Clone 30.12E belongs to the UniGene cluster Mm.136892. This clone maps on chromosome 1E3, and by bioinformatic analysis it was not possible to identify any ORFs

displaying sequence similarity to known proteins. The ESTs sequences belonging to Unigene cluster 136892 are derived from foetal liver. kidney and retina libraries.

DNA binding.

Clone 22.10C corresponded to the Zfp131 gene, encoding for a zinc finger protein. To date there is no information about this gene, and it is possible only to hypothesize its transcriptional role in the process of differentiation.

Transporter.

Clone 9.4G corresponds to Slc16a4, a monocarboxylate transporter. Evidence suggest that lactate could be the preferential energy substrate transferred from astrocytes to neurons, and such a process implies the presence of specific monocarboxylate transporters on both cell types. In recent work it was demonstrated that isoforms of the monocarboxylate transporter family were expressed in cultures of mouse cortical astrocytes and neurons (Debernardi et al., 2003). These findings are in line with the up-regulation of Slc16a4 in the differentiated cells.

Calcium binding.

Clone 26.3G corresponded to Reticulocalbin and clone 28.1B to Calponin 3 acidic. Reticulocalbin is a luminal protein of the endoplasmic reticulum (Ozawa and Muramatsu, 1993). The protein has six high-affinity Ca^{2+} binding motives, the EF-hand. Calponin is a thin filament-associated protein in smooth muscles, and it interacts with several Ca^{2+} binding protein in a Ca^{2+}-dependent manner (Szymanski and Tao, 1993). The one hundred amino acid Calponin homology (CH) domain is responsible for the binding of both signalling and cytoskeletal proteins (Gimona et al., 2002). The Ca^{2+} binding activity is probably of primary importance in differentiating and differentiated cells, that are assembling the neuronal circuitry architecture.

Cell-cell interaction.

Clone 32.4D corresponds to Connexin 43, which is the highest expressed of the *Tess* clones in differentiated cells. This protein is involved in gap junction formation, which are channels that span two plasma membranes and are known to link astrocytes through the connexin proteins, including Connexin 43. During development, Cx43 transcripts were found in discrete spatially restricted domains of the embryo, including the brain. The differential localization of Cx43 transcripts has been associated with the developmental processes mediated by inductive interactions, in the eye, optic vesicle, kidney, and the development of the branchial arches (Ruangvoravat and Lo, 1992).

Conclusions

A first point that needs to be underscored is the use of a specific subtractive cDNA library, the *Tess* library (Telencephalic Embryonic Subtractive Sequences (Porteus et al., 1992)), containing genes that are preferentially expressed at E14.5 in the developing telencephalon, and specifically involved in the embryonic processes of neural cell proliferation, migration and differentiation. This cDNA library has been generated by subtracting away most of the transcripts present in adult brain. Since neural stem cells in the adult brain are a very small population compared to the other cells that constitute a brain, it may be more difficult to select specific developmental genes using different arrays such as the ones that include the "entire" mouse transcriptome. The rationale of such an approach is that, using the *Tess* cDNA chip, it is possible to identify rare or still unknown transcripts specifically expressed in regions where neurogenesis occurs, having a role in proliferation and differentiation of adult neural stem cells. The results obtained by the microarray experiments confirmed this approach: the clones that were identified as differentially expressed during the process of differentiation are potentially significant because of their sequence homology, function or simply because of their expression pattern. For instance, by looking at the functional classification (Table 15, 17, 19) and distribution of the *Tess* genes it was possible to verify that:

The stem/progenitor cell populations are characterized by a high level of expression of genes involved in

- Cell cycle,
- Transcription,
- Chromatin remodelling,
- DNA repair,
- Detoxification.

Whereas the differentiated cell population is characterized by an up-regulation of genes involved in

- Transport,
- Calcium binding,
- Cell-cell contact,
- Cytoskeleton organization.

This functional distribution is interesting, since it provides two classes of genes supposed to play different roles in undifferentiated and differentiated cells. The first set of genes, preferentially expressed in undifferentiated cells, may be related to the maintenance of the "stem cell phenotype"; for example the genes involved in cell cycle and DNA repair may be involved in self-renewal, an unique characteristic of stem cells. The other genes, such as those involved in transcription and chromatin remodelling, may be important in the processes of lineage commitment. Remarkably, the finding of Mest/Peg1 as the most differentially expressed gene in undifferentiated cells, underlines the role of detoxification in the maintenance of a stem cell population. On the contrary, differentiated cells have to absolve more specialized tasks (axonal connexion, branching, vesicular release, etc.). Thus, the genes preferentially expressed in this population fall into classes that are specific of the neural or glial phenotype (cell-cell contact, cytoskeleton organization, etc.).

Certainly the aim of the study was not to perform a comprehensive transcriptional "genome-wide" profiling, but the subtractive library and array of *Tess* cDNAs allowed me to concentrate the analysis on the potentially significant genes in stem cell biology. Thus the specificity of this cDNA chip has not beeen considered a limitation, but instead an advantage since it permits a faster selection and a more direct focus on the genes of interest.

MATERIALS AND METHODS

Microarray production

Pcr amplification and clone preparation

In order to realize a specific mouse telencephalic cDNA microarray starting from the telencephalic expressed sequences library (*Tess* library) the inserts of 1026 different *Tess* clones were amplified by PCR with 2μl from an overnight bacterial culture with vector primers (T3, 5'-TTAACCCTCACTAAAGGGAAC-3', and T7, 5'-GTAATACGACTCACTATAGGG-3') in a total volume of 50μl in 96-well reaction plates (Thermowell 96 Well Plate, Corning, The Netherlands). PCR was performed by preheating the samples at 94°C for 10 min, 35 cycles at 94°C for 30 s, 53°C for 30 s and 72°C for 2 min, followed by 5 min of extension at 72°C. An aliquot of the PCR products was electrophoresed on a 1% agarose gel to monitor the success of the reaction.

For efficient binding of the amplified clone inserts to the slides, it is essential to remove unincorporated nucleotides and primers from the reaction products. The PCR products were cleared by QIAquik Multiwell PCR Purification Kit (Qiagen, Germany) using QIAvac 96 vacuum Manifolds (Qiagen, Germany). The PCR products were eluted in 50μl of H_2O in to 96 well plates (96 well round prolypropylene, Nunc, USA). I found that PCR products printed using 50% DMSO as printing buffer provides the best substrate for hybridization, allowing me to achieve a high signal to noise ratio. For this reason the eluted products were vacuum-dried and resuspended in 10μl of 50% DMSO. Plates containing the purified PCR products were then sealed using a cap mat (Adhesive PCR Foil Seals, #AB-0626, Abgene, UK) and stored at 4°C.

Controls are important for microarray analysis because of the complexity of the experiment: in order to allow a careful analysis of the data, 22 artificial genes designed

from yeast intergenic region were added to the cDNA array (Lucidea Universal ScoreCard, #RPK3161, Amersham Biosciences, UK).

Array printing

The plates containing the PCR products and the controls were loaded into the arrayer (SpotArray 24, Perkin Elmer, USA) using Stealth Micro Spotting Pins (Telechem, USA), setting the temperature to 22°C and the relative humidity to 50%. Twenty-five "pre-prints" were done onto the blotting substrate before printing on the microarray substrate (Super Amine slides, Telechem, USA), in order to obtain a good spot morphology. The clones were printed in four replicates onto the microarray substrate, using the following specifications: the nominal spot diameter was 150 μm, the spot spacing 300μm, the printing approach velocity 15mm/sec, the departure 10mm/sec, the overtravel 500μm, the printing dwell time 25 mSec, the sample load overtravel 500μm and the sample load dwell 1000 mSec.

After the printing procedure the slides were baked at 80°C in a drying oven, and the printed substrates were rinsed twice in 0.1% SDS and once in dH_2O for 2 min, each wash at room temperature to remove unbound DNA. After washing the processed substrates were dried by centrifugation for 2 min at 800X g. Printed slides were stored in a light-tigh box in a bench-top dessicator at room temperature. Following the printing procedure one slide of the batch was controlled using a fluorescent DNA-dye (Vistra green, #RPN 5786, Amersham Bioscience, UK): the slide was scanned at 670nm using a confocal laser scanner (Scannarray Express, Perkin Elmer, USA) producing a TIFF image, and the image was analysed to control the presence of the spots and their morphology.

Probe preparation and Hybridization

Labelling of total RNA was performed using a novel procedure: the dendrimer technology (3DNA Submicro Expression Array Detection Kit, #A100731/100741 Genisphere, USA).

This procedure required a 2-step protocol: first there was the reverse transcription (RT) of the total RNA using special RT primer oligo, then the cDNA and the fluorescent 3DNA reagent were hybridized to the array in succession.

RNA Extraction

RNA from cells and tissues was extracted using a method based on guanidinium lysis and phenol-chloroform extraction (ToTALLY RNA, Ambion, USA). Briefly, samples are lysed in a guanidinium based lysis solution and are then extracted sequentially with Phenol:Chloroform:IAA and Acid-Phenol:Chloroform. The RNA is then precipitated with isopropanol and resuspended in DEPC water.

To assess the concentration and purity of RNA, an aliquot was used to read the absorbance using a spetrophotometer at 260nm and 280nm, and the overall quality of the RNA preparation was checked by electrophoresis on a 1% denaturing agarose gel.

Reverse Transcription

Total RNA (from 1 to 10μg, depending on the samples) from two different cell populations or two different tissues, 3μl of primers (0.2pmole) and DEPC water to a final volume of 10μl were combined in a RNase-free 0.5mL tube, incubated for 10 min at 80°C and immediately transfer to ice. In a separate microtube the following reagents were combined: 4μl of 5X RT buffer (Promega, USA), 1μl of 10mM each dNTP mix, (Promega, USA), 2μl 0.1M dithiotreitol (Promega, USA), 1μl of 1u/μl ribonuclease inhibitor (Promega, USA) and 1μl of reverse transcriptase enzyme (Improm-II, Promega, USA). This reaction mix was added to the RNA-primer mixture and incubated at 42°C for two hours. The reaction was stopped by adding 3.5μl of 0.5M NaOH/50mM EDTA and incubated at 65°C for 10 min to denature the DNA/RNA hybrids. Then the reaction was neutralized with 5μl of 1M Tris-HCl, pH 7.5.

Concentration of cDNA

For dual channel expression analysis the cDNAs were combined in one tube. Three µl of 5mg/ml linear acrylamide (Genisphere, USA) were added to the combined cDNA to act as a co-precipitant. In order to precipitate the cDNA, 6µl of 5M NaCl and 250µl of 100% ethanol were added, and the sample was incubated at –20°C for 30 min, centrifugated at 11000g for 30 min, washed with 300µl of 70% ethanol and eventually resuspended in 18µl of nuclease free water.

Hybridization of cDNA to the array

In order to assemble the hybridization mix, these additional reagents were added to the concentrated cDNA: 20µl of 2X hybridization buffer (50% formamide, 8X SSC, 1% SDS, 4X Denhardt's solution) and 2µl of dT blocking reagent (LNA dT blocker, Exiqon, USA) to a final volume of 40µl. The hybridization mix was incubated first at 80°C for 10 min and then at 48°C for 20 min. Before the hybridization the array was incubated in 5X SSC, 0.1% SDS, 1% BSA at 48°C for 45 min in order to reduce the background. Then the array was washed in H_2O for 2 min and dried by centrifugation at 800Xg for 3 min. Before adding the hybridization mix, the array was pre-warmed at 48°C to reduce the background. A 22x40 coverslip (Hybri-slip, Grace bio-labs, USA) was then added to the array and the incubation was performed overnight in a dark humidified chamber at 48°C.

After hybridization the slide was washed briefly four times to remove unbound cDNA. A first wash was performed at 50°C for 7 min with 2XSSC and 0.2% SDS, a second one at room temperature for 7 min with 1XSSC, and a third one at room temperature for 7 min with 0.1X SSC. The array was then washed for 2 min at room temperature in 95% ethanol to fix the cDNA molecules to the probes. The array was finally dried by centrifugation at 800Xg for 3 min.

cDNA labelling

To a 1.5 ml tube the following components were added to assemble the hybridization mix: 22.5 µl of 2X hybridization buffer (50% formamide, 8X SSC, 1% SDS, 4X Denhardt's solution), 2.5 µl of each Cy3 and Cy5 3DNA capture reagent (Genisphere, USA), 1 µl of High-End differential Enhancer (Genisphere, USA) and 16.5 µl of nuclease free water to a final volume of 45 µl. The hybridization mix was incubated first at 80°C for 10 min and then at 48°C for 20 min before adding the hybridization mix the array was pre-warmed at 48°C to reduce the background. A 22x45 coverslip (Takara spaced cover glass, Takara, Japan) was then added to the array and the incubation was performed for 3 hours in a dark humidified chamber at 48°C.

After hybridization the slide was washed briefly several times to remove unbound 3DNA molecules, and the washes were performed as described above in the cDNA hybridizations, excluding the ethanol wash. All the washes were performed in the dark to avoid photobleaching and fading of the fluorescent dyes. To reduce fading of Cy5 it was also beneficial to add DTT in the first two washing buffers at a final concentration of 1mM. The array was finally dried by centrifugation at 800Xg for 3 min.

Data collection, normalization and analysis

Differential gene expression is assessed by scanning the hybridized arrays using a confocal laser scanner capable of interrogating both the Cy3 and Cy5-labeled probes and producing separate TIFF images for each (Perkin Elmer, USA). These images were then used to normalize and quantitate the data.

Slide scanning

Hybridized slides were scanned with ScanArray express (Perkin Elmer, USA). It was important to avoid excess scans, as the dyes may photobleach from exposure to the scanner light source. For this reason the array was subjected to an initial scanning using a low laser

power (80% for Cy3 and 70% for Cy5) and low PMT (70% for Cy3 and 65% for Cy5) with a resolution of 30μm, to get rapidly an image for both dyes. This image was used to balance the laser power and PMT according to the artificial genes from yeast intergenic regions spotted onto the array: the signal intensity for these spot had to be equal in both channels. After this initial scanning high resolution images (5μm) were collected: at least two images for each dye were collected using different laser power and PMT in order to carefully analyze the results.

Image processing

Image processing is required to extract measures of transcript abundance for each gene spotted on the array from the laser scan images.

A first image processing stage was the identification of the arrayed genes that have to be distinguished from spurious signals that can arise due to hybridization artefacts. A grid built by the scanner software (using a .gal file generated by the spotter) was used to locate the spot and quantitate exactly the signal for each spot. It was important to correct manually the grid to avoid false location procedure by the software: generally, problems of grid spot location are coupled with bad estimation of the fluorescent signal and background. Following spot identification and local background determination, the background-subtracted hybridization intensities for each spot were calculated.

Data quantification and normalization

After the grid is adjusted the computer software determined the centre for each spot, and determined the corresponding circle. The circle diameter was estimated separately for each spot on the microarray. The quantification task then constructed masks for the spot and the background. A mask is a pixel-by-pixel map that indicates the property of each pixel.

In order to generate a list showing the signal intensities for each gene, I used all the spots having signal intensity above the signal generated by negative control spots, that were

DNA fragments derived from unrelated species (Lucidea Universal ScoreCard, Amersham Biosciences, UK). These spots are used to monitor the specificity of hybridization and to set the detection limit.

Microarray data are subject to multiple sources of variation. Normalization is needed to identify and remove systematic sources of variation, such as differing dye labelling efficiencies and scanning proprieties. I used a new normalization method for reducing such variability, the LOWESS method (Locally Weighted Scatter Plot Smoothing: http://stat-www.berkeley.edu/users/terry/zarray/TechReport/589.pdf). In particular this normalization task was useful for the spots characterized by low signal intensities: often normalization to the total was prone to produce a bending in the scatter plot for the low expressed genes.

Sequence analysis

Clone growth

The clones found consistently differentially regulated by microarray hybridizations were inoculated in to 5ml of LB medium supplemented with 50μg/ml ampicillin and incubated for 16 hours at 37° and 200 rpm in a shaking incubator. Mini-preparations of plasmid DNA were performed using NucleoSpin Plasmid (Macherey-Nagel, Germany). An aliquot of the plasmid DNA was used for sequencing and for digestion in order to obtain linearized DNA used in the RNA *in situ* hybridizations.

Database searching

The obtained sequences were used to verify the identity of the clone of interest: a Comp-Blast analysis was performed (http://tigemania.tigem.it/compblast/) using the *Tess* sequences as a target database. This procedure was followed because of the possibility of picking errors during the procedure of clone growth.

Then the sequence of each individual clone was analysed using BlatN and BlastX homology searches against the non-redundant, dbEST, and UniGene database. When the clones didn't show any match in these databases, the sequences were further analysed against mouse and human genomic database using the UCSC Genome Bioinformatics Site (http://genome.ucsc.edu/) .

The analysis for the putative antisense sequence for *Tess* clone 31.5E was performed using the ANTIHUNTER (http://bio.ifom-firc.it/ANTIHUNTER/). It is a new tool to detect potential EST antisense transcripts within a given genomic region. The use of this program allows BLASTING the EST database using as a query an annotated genomic region (i.e., a genomic region in which gene location and strand occurrence is reported) and to parse the BLAST output for the presence of EST transcripts that are antisense transcribed with respect to the user supplied gene list. False-positive rate, deriving from erroneous EST strand annotation, is reduced by incorporating a check for the presence of splicing site consensi and for the presence of a polyA tail in 3' ESTs.

When a putative novel gene was found, the corresponding protein sequence was analysed by BlastP searching against protein databases. In order to characterize the presence of conserved domains, the protein sequence was subjected to an analysis using SMART (a Simple Modular Architecture Research Tool) that allows the identification and annotation of genetically mobile domains and the analysis of domain architectures. More than 500 domain families found in signalling, extracellular and chromatin-associated proteins are detectable. These domains are extensively annotated with respect to phyletic distributions, functional class, tertiary structures and functionally important residues. Each domain found in a non-redundant protein database as well as search parameters and taxonomic information are stored in a relational database system (http://smart.embl-heidelberg.de/) (Schultz et al., 2000).

Clustering

The cluster of the genes differentially expressed in neural stem cells, progenitor cells and differentiated cells was made using the CAGED software (http://genomethods.org/caged). The term CAGED stands for Cluster Analysis of Gene Expression Dynamics. It is a computer program performing Bayesian clustering on temporal gene expression data, which identifies the most probable set of clusters given a set of time series.

The analytical task is to partition the genes into clusters sharing a similar behaviour over the different time points (in this case: Neural stem cells, progenitor cells, and differentiated cells). In order to use this program a file had to be generated. This was a database in ASCII tab delimited format with 4 columns. The first was a description of each gene in the database (the clone name), the second the neural stem cells values, considered as 1, the third and the fourth reported the expression values of the *Tess* clone in progenitor cells and differentiated cells compared to stem cells.

Adult Neural Stem Cells culture

Collection of tissue

The mice were anaesthetized by intraperitoneal injection of pentobarbital (120mg/Kg) and sacrificed by cervical dislocation. Tissues from two or three mice are generally pooled to start a culture. Using large scissors the head was cut off just above the cervical spinal cord region and washed with 70% ethanol. After removing the skin and the skull the brain was removed. To dissect the forebrain subventricular region, the dish containing the brain was put under the dissecting microscope (10x magnification). The brain was positioned flat on its ventral surface and a coronal cut was made just behind the olfactory bulbs. A second cut was made to dissect an approximately 2mm thick slice embodying the lateral ventricles. The tissue was dissected under 25x magnification to recover the thin layer of tissue surrounding the ventricles, excluding the striatal parenchyma and the corpus callosum.

Dissociation of brain tissue

The dissected tissue was cut using fine scissors into small pieces. The pieces were transferred into plastic conical bottom 15ml tubes and 14ml of papain/DNAse solution was added. The tubes were incubated at 37°C for 30-60 minutes on a rocking platform. At the end of the enzymatic incubation the tissues were centrifugated at 110g for 10 minutes. After centrifugation the supernatant was removed and 3ml of ovomucoid solution (0.7mg/ml in culture medium) was added. Using a Pasteur pipette the tissues were dissociated by triturating 20-30 times. The cells were collected by centrifugation (110g for 10 minutes). Next the supernatant was removed, 5ml of culture medium was added, and the cells were centrifugated again (110g for 10 minutes). The supernatant was discarded and the cells were resuspended in 0.5ml of culture medium. An aliquot was counted in a haemocytometer.

Culture propagation

The cells were seeded at a density of 3500/cm^2 in culture medium in untreated 6-well tissue culture dishes (2ml volume) and incubated at 37°C, 5% CO_2 in a humidifier incubator. The cells proliferated to form spherical clusters and lifted off as they grew larger. The growth medium was DMEM/F12 with 20ng/ml of both epidermal growth factor (EGF) and fibroblast growth factor (FGF-2). Individual primary spheres were mechanically dissociated, and single cells were plated in growth medium. To obtain progenitor cells, the spheres were transferred onto matrigel-coated new dishes in the presence of 20ng/ml of FGF-2 for 48hr culture. To obtain differentiated cells, the culture was successively shifted to control medium (DMEM/F12 without growth factors) containing 2% foetal bovine serum (FBS) for 6 days.

Data validation

RT-PCR

The same RNA used for microarray hybridization was used to perform retro-transcription. One microgram of total RNA was transcribed using 150ng of random hexamers and 200 units Superscript II RT (Invitrogen, UK) in a 20μl reaction at 42° for 60 min and 2μl of the cDNA was used in PCR amplifications using pairs of the following primers:

for mouse β-actin:

Forward: 5'-GGCATCGTGATGGACTCCG-3'

Reverse : 5'-GCTGGAAGGTGGACAGCGA-3'

for *Tess* 13.6H

F: 5'-GCATGTGTCCCGTTTCCCGT-3'

R: 5'-CAACTAAGTCAGATCCTGACTGC-3'

For *Tess* 12.6B

F: 5'-GGGAATCCCAGCTCTCCCTCCA-3'

R: 5'-TCCTCCTTGGTGATGAGTGGGTA-3'

For *Tess* 21.12E

F: 5'-TCTAGATAACCAGGCGGTGGC-3'

R: 5'-TCCACTGTCTGCAGCTCCTCTG-3'

For mouse Pcna

F: 5'-TTGTCGCTGTAGGCCTTCGTTC-3'

R: 5'-GCTGGCATCTCAGGAGCAATCT-3'

For AK032537

F: 5'-GCTTCAAATTGGATGGCACTGC-3'

R: 5'-CTTAGGCAGAGTGAAGCCTGTAG-3'

For AK038694

F: 5'-GACTTGGTCCAAGCTGCGGAG-3'

R: 5'-CTTAGGCAGAGTGAAGCCTGTAG-3'

For human Mest/Peg1 original isoform

F: 5'-ATGGGATAACGCGGCCATGGTG-3'

R: 5'-ATAGTGATGTGGTCTCGGTTTGTCACTG-3'

For human Mest/Peg1 alternative isoform

F: 5'-AGTCCTGTAGGCAAGGTCTTACCTG-3'

R: 5'- ATAGTGATGTGGTCTCGGTTTGTCACTG-3'

for human β-actin:

F: 5'-GAGCACAGAGCCTCGCCTTTG-3'

R: 5'-GGTGTTGAAGGTCTCAAACATG-3'

PCR amplifications were performed as a standard 50μl reaction (2-2.5 μl cDNA template, 50pmol each primer, 200μM dNTPs, 50 mM Kcl, 10mM TrisHCl ph 8.3, 1.5 mM $MgCl_2$, 0.5 units AmpliTaq polymerase, Perkin Elmer).

35 cycles of amplification at 94° for 30s, 60° - 63° for 30s, 72° for 30s-1min were carried out, followed by a final extension step at 72° for 5 min. Primers for β-actin were added after 10-15 cycles. All PCRs were carried out in a Geneamp PCR system 9700 (Perkin Elmer, USA). An aliquot of the PCR products were electrophoresed on a 1% agarose gel to monitor the success of the reaction.

Cell sorting

In order to check human Mest/Peg1 expression in haematopoietic stem cells, sorted cell populations were used to purify RNA.

Surface staining of bone marrow mononuclear cells was performed using PE-conjugated anti-$CD34^+$ (Becton Dickinson, San Jose, CA) and TC-conjugated anti CD38 (Becton Dickinson, San Jose, CA). Cell sorting was performed using FACStar (Becton Dickinson, San Jose, CA). A lymphoblast-sorting gate was established for both forward and side

scattering. Then gates displaying PE fluorescence and/or TC fluorescence were generated, and cells were sorted. Immediately after the sorting the RNA was extracted in order to perform semi-quantitative RT-PCR.

In situ hybridization

The digestion of the plasmid DNA was performed using the following procedure: 10μg of DNA was digested in 60μl using 3μl (10u/μl) of ClaI as enzyme (Promega, USA) in 1X digestion buffer and incubated at 37°C for 3 hours. After this incubation an aliquot of the digestion was electrophoresed on a 0.8% agarose gel to monitor the success of the reaction. The DNA was extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and then with an equal volume of chloroform/isoamyl alcohol. The DNA was then ethanol precipitated with 0.3M sodium acetate and 2.5 volumes of 100% ethanol, centrifugated at 13000g for 10 min, washed with 75% ethanol and resuspended in 10μl of RNAse free water. In order to quantitate the linearized DNA, 1μl was electrophoresed on a 0.8% agarose gel.

The *in vitro* transcription was performed in 20μl with 20 units of T3 RNA polymerase (Promega, USA) in 1x transcription buffer, 40 units of RNase block (Promega, USA), and 10mM DTT. The reaction was incubated at 37°C for 2 hours, then ethanol precipitated with 0.3M sodium acetate and 2.5 volumes of 100% ethanol, centrifugated at 13000g for 10 min, washed with 75% ethanol and resuspended in 20μl of RNAse free water. In order to check the RNA integrity, 1μl was electrophoresed on a 0.8% agarose gel, and then 20μl of deionized formamide was added. The RNA was stored at –80°C.

The sections were fixed with 4% paraformaldheide, briefly washed three times with 1x phosphate buffer saline (PBS), incubated in proteinase K (1μg/mL) and post-fixed in 4% paraformaldehyde. In order to block endogenous phosphatases, the sections were incubated in 0.1M Triethanolamine and 0.25% Acetic Anhydride. Finally the sections were permeabilized in 1% Triton in 1x PBS and then briefly washed in 1x PBS.

Pre-hybridization was performed in 500µl of Hybridization buffer (50% formamide, 5X Denhardt's, 5x SSC, 250µg/mL yeast tRNA, 500µg/mL salmon sperm DNA) for 2 hours at room temperature. Then the labelled riboprobes (about 200-400 ng/mL) were heated at 80°C for 5 min, placed immediately on ice, mixed with the hybridization mix at 72°C and finally placed on sections. The incubation was performed at 72°C for 16 hours.

The sections were then washed several times at 72°C with 0.2X SSC, and then pre-incubated with 10% of goat serum in 100mM Tris-HCl pH 7.5 and 150mM NaCl at room temperature for one hour. The anti-digoxigenin Fab fragments (Boeheringer, Germany) was added to the sections in 100mM Tris-Hcl pH7.5, 150mM NaCl and 1% of goat serum and then incubated at 4°C for 16 hours. The sections were then washed several times in 100mM Tris-Hcl pH7.5 and 150mM NaCl at RT. To detect the signal the sections were incubated in 100mM Tris-HCl pH9.5, 100mM NaCl, 50mM $MgCl_2$, 450µg/µl Nitro Blue Tetrazolium (NBT), 175µg/µl (5-bromo-4-hloro-3-indolyl phosphate) BCIP for 6 hours to 3 days in the dark at RT. When the signal was strong enough the reaction was stopped with TE pH8 at room temperature for 5 min, post-fixed with 4% PFA and rinsed with PBS.

The slides were air dried overnight, placed in xylene twice for one minute and finally mounted and analysed at the microscope.

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